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(54) Title: KRINGLE DOMAINS OF PLASMINOGEN, CAPABLE OF MODULATING ANGIOGENESIS IN VIVO

(57) Abstract

The present invention relates to compositions and proteins capable of regulating the endothelian cell proliferation as well as various uses thereof. The compositions and proteins are characterised by inclusion of amino acid sequences associated with mammalian Kringles 1, 2 and/or 3 together with that of Kringle 5 or consist of anti–angiogenically active peptide fragments or protected derivatives thereof. The protein according to the invention has a molecular weight of approximately 50–75, such as 50–60 kDa, preferably about 55 kDa, and has an amino acid sequence, which is substantially similar to that of a plasminogen fragment comprised of Lys 78–Arg 530 of a plasminogen molecule.

KRINGLE DOMAINS OF PLASMINOGEN, CAPABLE OF MODULATING ANGIOGENESIS IN VIVO.

Technical field

The present invention relates to novel endothelial cell proliferation inhibitors. The inhibitors are extraordinarly potent in inhibiting angiogenesis related diseases and modulating angiogenic processes. In addition, the present invention relates to methods of treating a human or animal having an angiogenic disease by administering an inhibitor according to the invention thereto.

Background

As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ, and involves endothelial cell proliferation. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development and formation of the corpus luteum, endometrium and placenta. The term "endothelium" means a thin layer of flat epithelial cells that lines serous cavities, lymph vessels and blood vessels.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells and supports the pathological da-

mage seen in these conditions. The diverse pathological disease states in which unregulated angiogenesis is present have been grouped together as angiogenic dependent or angiogenesis associated diseases.

The hypothesis that tumor growth is angiogenesis-dependent was first proposed in 1971. (Folkman J., Tumor angiogenesis: Therapeutic implications. *N. Engl. Jour. Med.* 285:1182-1186, 1971). In its simplest terms it states: "Once tumor "take" has occurred, every increase in tumor cell population must be preceded by an increase in new capillaries converging on the tumor. Tumor "take" is currently understood to indicate a prevascular phase of tumor growth in which a population of tumor cells occupying a few cubic millimeters volume, and not exceeding a few million cells, can survive on existing host microvessels. Expansion of tumor volume beyond this phase requires the induction of new capillary blood vessels. For example, pulmonary micrometastases in the early prevascular phase in mice would be undetectable except by high power microscopy on histological sections.

Examples of the indirect evidence which support this concept include:
(1) The growth rate of tumors implanted in subcutaneous transparent chambers in mice is slow and linear before neovascularization, and rapid and nearly exponential after neovascularization. (Algire GH, et al. Vascular reactions of normal and malignant tumors *in vivo*. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants.; *J Natl. Cancer Inst.* 6:73-85,1945).
(2) Tumors grown in isolated perfused organs where blood vessels do not proliferate are limited to 1-2 mm^3 but expand rapidly to > 1000 times this volume when they are transplanted to mice and become neovascularized. (Folkman J, et al., Tumor behavior in isolated perfused organs: *In vitro* growth and metastasis of biopsy material in rabbit thyroid and canine intestinal segments. *Annals of Surgery* 164:491-502, 1966).
(3) Tumor growth in the avascular cornea proceeds slowly and at a linear rate, but switches to exponential growth after neovascularization. (Gimbrone, M.A., Jr. et al., Tumor growth and neov ascularization: An experimental model using the rabbit cornea. *J. Natl. Cancer Institute* 52:41-427, 1974).

(4) Tumors suspended in the aqueous fluid of the anterior chamber of the rabbit eye, remain viable, avascular and limited in size to < 1 mm^3. Once they are implanted on the iris vascular bed, they become neovascularized and grow rapidly, reaching 16,000 times their original volume within 2 weeks. (Gimbrone MA Jr., et al., Tumor dormancy *in vivo* by prevention of neovascularization, *J. Exp. Med.* 136:261-276).

(5) When tumors are implanted on the chick embryo chorioallantoic membrane, they grow slowly during an avascular phase of >72 hours, but do not exceed a mean diameter of 0.93 + 0.29 mm. Rapid tumor expansion occurs within 24 hours after the onset of neovascularization, and by day 7 these vascularized tumors reach a mean diameter of 8,0 + 2,5 mm. (Knighton D., Avascular and vascular phases of tumor growth in the chick embryo. *British J. Cancer*, 35:347-356,1977).

(6) Vascular casts of metastases in the rabbit liver reveal heterogeneity in size of the metastases, but show a relatively uniform cut-off point for the size at which vascularization is present. Tumors are generally avascular up to 1 mm in diameter, but are neovascularized beyond that diameter. (Lien W., et al., The blood supply of experimental liver metastases. II. A microcirculatory study of normal and tumor vessels of the liver with the use of perfused silicone rubber. *Surgery* 68:334-340,1970).

(7) In transgenic mice which develop carcinomas in the beta cells of the pancreatic islets, pre-vascular hyperplastic islets are limited in size to < 1 mm^3. At 6- 7 weeks of age, 4- 10% of the islets become neovascularized, and from these islets arise large vascularized tumors of more than 1000 times the volume of the pre-vascular islets. (Folkman J, et al., Induction of angioaenesis during the transition from hyperplasia to neoplasia. *Nature* 339:58-61,1989).

(8) A specific antibody against VEGF (vascular endothelial growth factor) reduces microvessel density and causes "significant or dramatic" inhibition of growth of three human tumors which rely on VEGF as their sole mediator of angiogenesis (in nude mice). The antibody does not inhibit growth of the tumor cells *in vitro*. (Kim K J, et al., Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth *in vivo*. *Nature* 362:841-844,1993).

(9) Anti-bFGF monoclonal antibody causes 70% inhibition of growth of a mouse tumor which is dependent upon secretion of bFGF as its only mediator of angiogenesis.

The antibody does not inhibit growth of the tumor cells *in vitro*. (Hori A, et al., Suppression of solid tumor growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. *Cancer Research*, 51:6180-6184, 1991).

(10) Intraperitoneal injection of bFGF enhances growth of a primary tumor and its metastases by stimulating growth of capillary endothelial cells in the tumor. The tumor cells themselves lack receptors for bFGF, and bFGF is not a mitogen for the tumor cells *in vitro*. (Gross JL, et al., Modulation of solid tumor growth *in vivo* by bFGF. *Proc. Amer. Assoc. Canc. Res.* 31: 79 , 1990).

(11) A specific angiogenesis inhibitor (AGM-1470) inhibits tumor growth and metastases *in vivo*, but is much less active in inhibiting tumor cell proliferation *in vitro*. It inhibits vascular endothelial cell proliferation half-maximally at 4 logs lower concentration than it inhibits tumor cell proliferation. (Ingber D, et al., Anaioinhibins: Synthetic analogues of fumagillin which inhibit angiogenesis and suppress tumor growth. *Nature*, 48:555-557.1990). There is also indirect clinical evidence that tumor growth is angiogenesis dependent.

(12) Human retinoblastomas that are metastatic to the vitreous develop into avascular spheroids which are restricted to less than 1 mm^3 despite the fact that they are viable and incorporate 3H-thymidine (when removed from an enucleated eye and analyzed *in vitro*).

(13) Carcinoma of the ovary metastasizes to the peritoneal membrane as tiny avascular white seeds (1-3 mm^3). These implants rarely grow larger until one or more of them becomes neovascularized.

(14) Intensity of neovascularization in breast cancer (Weidner N, et al., Tumor angiogenesis correlates with metastasis in invasive breast carcinoma. N. Engl. J. Med. 324:1-8,1991, and Weidner N, et al., Tumor angioaenesis: A new significant and independent prognostic indicator in early-stage breast carcinoma, *J Natl. Cancer Inst.* 84:1875-1887, 1992) and in prostate cancer (Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *American Journal of Pathology*, 143(2):401-409,1993) correlates highly with risk of future metastasis.

(15) Metastasis from human cutaneous melanoma is rare prior to neovascularization. The onset of neovascularization leads to increased thickness of the lesion and an increasing risk of metastasis. (Srivastava A, et al., The prognostic significance of tumor vascularity in intermediate thickness (0.76-4.0 mm thick) skin melanoma. *Amer. J. Pathol.*133:419-423,1988)

(16) In bladder cancer, the urinary level of an angiogenic peptide, bFGF, is a more sensitive indicator of status and extent of disease than is cytology. (Nguyen M, et al., Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in urine of bladder cancer patients. *J. Natl.Cancer Inst.* 85:241-242,1993).

Thus, it is clear that angiogenesis plays a major role in the metastasis of a cancer. If this angiogenic activity could be repressed or eliminated, or otherwise controlled and modulated, then the tumor, although present, would not grow. In the disease state, prevention of angiogenesis could avert the damage caused by the invasion of the new microvascular system. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

Accordingly, within this field, there is a strong need for compositions and methods by which endothelial cell proliferation, such as the unwanted growth of blood vessels, especially into tumors, may be inhibited. There is also a need for methods for detecting, measuring and localizing such compositions. Such compositions should be able to overcome the activity of endogenous growth factors in premetastatic tumors and prevent the formation of the capillaries in the tumors, thereby inhibiting growth of the tumors. In addition, the compositions, fragments of such compositions and antibodies specific to said compositions should be able to modulate the formation of capillaries in other angiogenic processes, such as wound healing and reproduction. Naturally, compositions and methods for inhibiting angiogenesis should preferably be non-toxic and produce few side effects. Also needed is a method for detecting, measuring and localizing the binding sites for the composition as well as sites of biosynthesis of the composition. The compositions and fragments of the compositions should be capable of being

conjugated to other molecules for both radioactive and non-radioactive labeling purposes.

It has been suggested to use a single protein consisting of the first four kringle regions, nos. 1-4, of plasminogen, also denoted angiostatin, as active agent in a composition for the above defined purposes; see US 5,639,725 incorporated herein by reference. However, the inhibitory effect of angiostatin on endothelial cell proliferation is too low to be satisfactory. Used as a medicament for human patients, angiostatin would have to be administered in kilograms to be effective, which of course would put severe limitations to the use thereof due to practical reasons. Another drawback lies in the amounts which would be necessary to produce due to said low effect, making such a product quite costly.

It has also been determined that proteins and peptides corresponding to all or part of the sequence of kringle 5 of plasminogen might also be so used, see US 5,854,221 and WO 97/41824 incorporated herein by reference. It is particularly suggested that fusion proteins of Kringle 5 with other proteins incorporating Kringle 4 might be used (see WO 97/41824, page 6, lines 7 to 18). Again the inhibitory effect is too low to be satisfactory for the reasons given above

Thus, in spite of the discovery of angiostatin and K5, there is still a strong need for a composition which fulfils the objects defined above.

Summary of the invention

The object of the present invention is to fulfil the above defined need. This is accomplished by the present invention, which relates to a protein and to protein and peptide containing compositions capable of modulating or regulating, e.g. inhibiting, the endothelial cell proliferation in *in vitro* and angiogenesis in *in vivo* assays. The invention also relates to any nucleic acid encoding such novel protein or combination. The various further aspects of the invention will be described in detail below with reference to the drawings.

The invention is particularly distinguished from the prior art in that the inventor has determined that by use of one or more of the angiostatin associated Kringles 1 to Kringle 3, or angiogenically active peptide fragments thereof, in association with Kringle 5 or its active peptide fragments or conjugates, it becomes possible to provide anti-angiogenesis treatment using far smaller quantities of agent than possible when using angiostatin or Kringle 5 type agents alone, rendering such treatment a more practical proposition. The inventor has particularly determined that angiostatin like agents and Kringle 5 like agents appear to exert their effect through separate pathways thus providing enhanced efficacy, ie. significant synergy, when used together in the same treatment, with a single agent incorporating a Kringle selected from Kringles 1, 2 and 3, or an active peptide fragment thereof, together with a Kringle 5 type component being preferred.

It is particularly surprising that a protein such as Kringle 1 (K1), which has very little activity itself, is capable of synergising with Kringle 5 (K5) such as to provide greater anti-angiogenic activity than either angiostatin (K1-4) or K5 when administered separately. The present invention is distinguished from that of the prior art in that it requires the presence of K1, K2 and/or K3, or active peptide fragments thereof, together with K5 or an active peptide fragment thereof, with result of a surprisingly potent anti-angiogenic activity.

<u>Brief description of the drawings</u>

Figure 1 A and B show a proteolytic human kringle 1-5 (K1-5) fragment; schematically (A) and purified on a gel (B).

Figure 2 show the anti-endothelial cell proliferation activity of K1-5 at low concentrations (A) and at high concentrations (B), while 2 C-D show the inhibition of capillary endothelial cell proliferation.

Figure 3 A-D shows the morphology of K 1-5 treated endothelial cells with bFGF alone (A); bFGF + K1-5 (10ng/ml) (B); bFGF + K1-5 (100ng/ml) (C); and bFGF + K1-5 (500ng/ml (D).

Figure 4 shows the synergistic suppression of endothelial cell growth by angiostatin (K1-4) and K5.

Figure 5 A-C shows the anti-angiogenic effect of K 1-5 according to the invention on the chick embryo chorioallantoic membrane (CAM).

Figure 6 shows suppression of primary tumor growth by K 1-5 according to the invention, comparing mice treated with K 1-5 with saline treated mice (A) and showing tumor volume with time (B).

Figure 7 A-D shows detection of microvessel density by immunohistochemical staining of tumor tissue sections with anti-vWF antibodies.

Figure 8 shows the synergistic effect of K1 and K5 and K1-3 and K5 on endothelial cell growth.

Detailed description of the invention

In a first aspect of the present invention there is provided a composition comprising one or more proteins or peptides which individually or together comprise (a) an amino acid sequence corresponding to that of one or more of Kringles 1, 2 and 3 of a mammalian plasminogen or anti-angiogenically active peptide framents or protected derivatives thereof and (b) an amino acid sequence corresponding to that of a peptide or protein selected from the group consisting of a mammalian plasminogen Kringle 5, its fusion proteins or anti-angiogenically active peptide fragments or protected derivatives thereof wherein

the total molecular weight of the protein and/or peptides (a) + (b) is no more than 50-75kDa, such as 50-70kDa, preferably 50-65kDa, as defined by reducing polyacrylamide gel electrophoresis.

Where the composition comprises only two Kringle (herein K) amino acid sequences, or comprises peptide fragments, the total molecular weight will be less than 40kDa, more preferably less than 30kDa, still more preferably less than 25kDa.

However, in the present context, it is noted that molecular weights have been determined by glycosylation of the protein. When Kringle 1-5 are glycosylated, the molecular

weight will be increased, and it is known that K1-5 contains at least one glycosylation site at Asn308. Further, under reducing conditions, the molecular weight of K1-5 will also be increased. Both glycosylation and reduction can be incomplete under the physiological conditions and electrophoresis conditions. Thus, the same protein can be present in two or more molecular weights, and even though 50-70kDa is mentioned above, under certain conditions, the molecular weight may be as high as 50-75 kDa.

Preferably the protein or active peptide fragment of amino acid sequence corresponding to that of one or more of K1, K2 and K3 of a mammalian plasminogen consists of a protein corresponding to the amino acid sequence of a mammalian plasminogen fragment K1, K2, K3, K1-2, K1-3, K2-3, K1-4, K2-4 or K3-4 and is of 45KDa or less as determined by reducing polyacrylamide gel electrophoresis. Conveniently K1-4 may be used as the component (a) as disclosed in US 5,639,725 is which incorporated herein by reference. However, for the purpose of decreased weight of actives while retaining efficacy, K1, K2 or K3 or their active peptide fragments will be preferred.

In a specific embodiment, for the purpose of decreasing molecular weight whilst retaining activity, this protein does not comprise Kringle 4.

The individual kringles and cDNAs encoding therefor may be provided by conventional means, eg. by methods described in Journal of Biological Chemistry 1996, Vol 271, No 46, Kringle Domains of Human Angiosatin, which is incorporated herein by reference. The individual kringle domains have been described, e.g. in Cao, Y., Ji, R., Davidson, D., Schaller, J., Marti, D., Söhndel, S., McCane, S., O'Reilly, M., Llinas, M., and Folkman, J., in J. Biol. Chem. 271, 29461-29467, 1996.

More preferably the peptide or protein having amino acid sequence corresponding to that of a mammalian plasminogen Kringle 5, its fusion proteins or an anti-angiogenically active peptide fragment or protected derivative thereof (b) is selected from the group of molecules comprising the amino acid sequence of mammalian plasminogen Kringle 5 Arg530 to Tyr534 and molecules comprising the amino acid se-

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quence of mammalian plasminogen Kringle 5 Pro150 to Tyr 156. Suitable peptides are disclosed in US 5,854,221 and WO 97/41824, derived from US Serial No 08/643,219 of 3rd May 1996 which are incorporated herein by reference. In the present context, reference is made to the enclosed sequence listings, wherein SEQ ID NO 1 discloses the amino acid sequence of human K1-5 while SEQ ID NO 2 discloses the sequence of mouse K1-5. The references herein to specific amino acids given herein may easily be translated to exact numbers in said sequence listings. However, as characterization of plasminogen kringle domains in the prior art have shown an essential homology between species, such as human, mouse, monkey, bovine and porcine species, in its broadest aspect, the present invention refer to the specifically given amino acid or its correspondence in other species, while specific embodiments of mouse and human could just as well be defined by sutable reference to said included sequence listing numbers. (For a reference to the characterization of K1-5 domains from various species, see e.g. Petros et al. *Eur. J. Biochem.* 170:549-63 (1988); Schaller et al. *Enzyme* 40:63-69 (1988); Ramesh et al. *Eur. J. Biochem.* 159:581-95 (1986); and Schaller et al. *Eur. J. Biochiem.* 149:267-278 (1985)).

Still more preferably the peptide or protein having amino acid sequence corresponding to that of a mammalian plasminogen Kringle 5, its fusion proteins or anti-angiogenically active peptide fragments or protected derivatives thereof is selected from the group of molecules comprising the amino acid sequence of mammalian plasminogen Kringle 5 Val454 to Ala543.

Most preferably the composition comprises only one (a single) anti-angiogenically active protein and that comprises an amino acid sequence corresponding to that of one or more of mammalian Kringles 1, 2 and 3 together with that of Kringle 5 in the same sequence. Alternatively the anti-angiogenically active peptide fragments of the selected Kringles may be substituted therefor in such single agent.

Conveniently the protein comprises plasminogen fragment K 1-3 joined to and Kringle 5 directly or by a spacer amino acid sequence. Such a spacer may be any sequence that

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the skilled in this field would consider suitable, e.g. in order to facilitate the preparation, and may be a short peptide, a polypeptide etc. The spacer is not intended to have any impact on the efficiency of the present composition, unless it improves the function by keeping said kringles apart in order to eliminate any possible undesired interaction between the two. In a specific embodiment, the protein comprises a spacer sequence corresponding to that of a mammalian Kringle 4. Further, in specific embodiments, any one of the kringle domains of the composition according to the invention may also or alternatively be kept suitably apart by use of spacers. However, for reason of reducing dose requirements in terms of weight, a composition comprising a single active protein, which comprises one of K1, K2 and K3 fused together with K5 is preferred. (For a reference regarding the term "spacer", see e.g. Cao, Y., Ji, R., Davidson, D., Schaller, J., Marti, D., Söhndel, S., McCane, S., O'Reilly, M., Llinas, M., and Folkman, J., in *J. Biol. Chem.* **271**, 29461-29467, 1996.)

From the point of view of ease of preparation from plasminogen, the most conveniently provided composition includes an anti-angiogenically active protein comprising an amino acid sequence corresponding to that of a mammalian plasminogen Kringles 1 to 5 Lys78 to Arg529 .

By a protected derivative is meant an N- and/or C-terminal protected protein or peptide, the protection being provided by conventional N- or C- terminal protecting groups, for example, such as acyl groups at the N-terminal and amide groups at the C-terminal, eg acetyl and amido respectively.

Accordingly, the invention particularly relates to compositions which are pharmaceutical or veterinary compositions, which are compositions of the invention which include one or more pharmaceutically acceptable carriers and/or excipients. The composition may be administered in a variety of unit dosage forms depending upon the method of administration,e.g parenteral, topical, oral or local administration, for prophylactic and/or therapeutic treatment. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and lozenges. A variety of carriers

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may be used, such as aqueous carriers, e.g. buffered saline etc. These solutions are free of undesirable matter. The compositions may also include pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents and the like, e.g. sodium acetate, sodium chloride, potassium chloride, calcium chloride etc. For parenterally administrable compositions, see e.g. Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980). A composition, or preparation, according to the present invention may be administered in a much lower dosage and the use thereof is thus superior to that of known angiostatin compositions. Consequently, the use of a pharmaceutical preparation comprising the Kringle proteins or peptide as described above when compared to use of angiostatin, is easier to administer due to the smaller amount needed, which smaller dose also results in a cheaper medicament. In one embodiment of the invention, the composition comprises a molecule or protein, which is capable of inhibiting cancer metastases. The half maximal concentration (EC50) of the agents, ie molecules according to the present invention for the inhibition of endothelial cell proliferation is typically about 50 pM, to be compared with the EC50 value for angiostatin, which is 130 nM. Thus, there is a 500-1000 fold difference between the effects of the compositions according to the invention and the effect of angiostatin, which is remarkable and highly surprising. Indeed, when the inhibitory effects of the first four kringle domains of plasminogen, angiostatin, were evaluated individually and combined, it was found that the most potent endothelial cell proliferation inhibition resulted when kringle region 4 was removed from angiostatin. (See The Journal of Biological Chemistry, 1996, vol 271, No 46: "Kringle Domains of Human Angiostatin", Cao et al.) . Thus, the general opinion within this area has been that a more potent fragment would result if kringle no 4 was removed from the protein. However, surprisingly, the present inventors have now shown that not only did the previously beleived inactive kringle 5 of plasminogen show effect, but, also, that the new molecule may also include the kringle 4, which has been known to lower the effect of the other angiostatin fragments. As mentioned above, the molecule according to the invention is also more stable in the human or animal body than the prior art compounds, probably due to a higher resistence against proteases and other enzymes present.

In other words, it exhibits a longer half-time, which is advantageous as it enables a less frequent administration, as well as lower amounts. Thus, for example, a K1-5 or a K1K5 fusion protein preparation according to the present invention is more convenient for the patient treated therewith and also less costly than the angiostatin or K5 compounds of the prior art.

In a second aspect, the present invention relates to isolated proteins and peptides corresponding in sequence to fusions of K1, K2, K3, K1-2, K1-3, K1-4, K2-3, K2-4, K3-4 or active peptide fragments thereof with K5 or an active peptide fragment thereof as described for inclusion in the compositions of the invention, these being capable of modulating endothelial cell proliferation *in vitro* and angiogenesis *in vivo*. In the case of K1-5 type molecules these will have an amino acid comprising at least about 50% of the sequence disclosed in SEQ ID NO. 1 or SEQ ID NO. 2 or an analogue thereof.

It will be realised that the fusion protein may have K5 at its N-terminal end or at its C-terminal end with respect to the other kringle sequence component and still retain anti-angeogenic activity. Conveniently the K5 will be C-terminal to the other kringle component.

Generally, for Kringle 1-5 type agents the protein according to the second aspect of the invention is comprised of at least about 50% of the amino acid sequence which the human kringle 1-5 sequence as disclosed in SEQ ID NO. 1 and the mouse kringle 1-5 as disclosed in SEQ ID NO. 2 has in common. In a more specific embodiment, the protein according to the invention comprises about 60-70% of the amino acid sequence which SEQ ID NO 1 an 2 has in common and more specifically essentially all of that sequence. In one particular embodiment, the protein according to the invention is comprised of essential parts the human amino acid sequence and in an alternative embodiment, the protein according to the invention is comprised of essentially all of the mouse amino acid sequence. It is to be understood that in all embodiments of the protein according to the invention, analogues and functional fragments thereof are also encompassed.

In one embodiment, that of K1-5, the protein according to the invention has a molecular weight of between about 50 to about 65 kilodaltons, as determined by non-reducing polyacrylamide gel electrophoresis. The molecular weight depends inter alia on whether the molecule has been glycosylated or not, which in turn depends on how it has been produced. The molecule according to the invention exhibits an amino acid sequence which is substantially similar to that of a plasminogen fragment comprised of Lys 78-Arg 530 of a plasminogen molecule. In the literature, the identity of the amino acids with which the plasminogen starts and ends have been debated. However, in the present context, the most important feature of the molecule according to the invention is that it possesses the advantageous angiogenic properties described herein. Thus, the sequence which the present molecule resembles may start with Lys 77 or Lys 78 and, correspondingly, it may end with Arg 529 or Arg 530. A preferred embodiment of the invention is a molecule, which more specifically has a molecular weight of about 50 to about 60 kilodalton, preferably about 53 to about 57 kilodalton and most preferred about 55 kilodalton. The plasminogen fragment according to the broad definition given above, i.e. comprised of the amino acid sequence Lys78-Arg 530 of plasminogen, is also denoted K1-5, as it comprises the first five kringle domains of plasminogen. Even though the fifth domain indeed has been known to exist before, the general opinion of the experts within this field has been that kringle no. 5 has not contributed to, or shared, the angiogenic properties of angiostatin. Thus, a fragment consisting of all the regions 1-5 of plasminogen has never been tested in total before as regards the inhibition of the growth of endothelial cells. The present inventor has now been able to disclose extremely promising results for such new fragments, or molecules exhibiting sequences substantially similar to such fragments, which compared to the prior angiostatin compositions quite surprisingly are superior in the present context. In addition to the advantages mentioned above, the molecule according to the invention is also more preferred than the prior angiostatin compounds, since the present molecule is larger. Consequently, the present molecule is more stable and therefore more favourable for use as a medicament and in medicinal compositions.

The present invention relates to a molecule as defined above, wherein the plasminogen fragment is similar to a fragment selected from the group consisting of human plasminogen, murine plasminogen, bovine plasminogen, Rhesus plasminogen and porcine plasminogen. The molecule, or protein, according to the present is capable of inhibiting endothelial cell proliferation in *in vitro* assays.

Another object of the present invention is the composition or molecule according to the invention, such as a two kringle K1K5 fusion protein, for use as a medicament. In addition, the invention also relates to the use of a composition according to the invention as defined above for the manufacture of a medicament for modulating, e.g. inhibiting, endothelial cell proliferation, for example for treating angiogenesis associated conditions or diseases, such as tumor growth, e.g. cancer, diabetes etc.

Another object of the present invention is a nucleic acid, such as a DNA or RNA, encoding a peptide, polypeptide or protein molecule according to the invention. A cDNA sequence which is complementary to such a sequence is also encompassed. Thus, further object of the invention is any nucleic acid which under stringent conditions hybridizes specifically to one of the above defined nucleic acids.

In the present context, the term hybridising specifically to refers to the binding, duplexing or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture of DNA or RNA. In the present context, the term "stringent conditions" refers to conditions, under which a probe will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. The one skilled in this field will easily choose the suitable conditions in the present context. Generally, stringent conditions are selected to be about 5^0C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, such as about 0.01-1.0 M, at a pH of about 7.0-8.3 and the temperature is between about 30^0C and 60^0C, depending on the length of the nucleoti-

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Monday 4
<--DD CL Ottawa Life Science Conference-->
	DD CL- Ottawa Life Science Conference
9:00-17:00	Analytical Meeting
9:00-10:30	QJ DBr - Visit from Mr. Karatsu Japan Technosearch
10:00-11:00	QJ-Schwarz
14:00-15:00	QJ TB- call Patrick Boyd
14:00-14:30	QJ TB-call Dr. Schteingart, Ferring
14:30-15:30	DCS BLA - Group Leader Meeting re: Status
14:30-15:00	

Tuesday 5
10:00-11:30	Senior Management Meeting
10:00-11:00	TM RL BLA KT FP - Quality meeting
12:00-14:00	DD - Luc Mainville RTP
14:30-15:00	DCS RL - Group Leader Meeting - re: Status
18:00-21:00	DD CL - HSBC Fifth Forecast Dinner

Wednesday 6
9:00-10:30	Chemistry Meeting
9:00-10:00	Pharmacology meeting
10:00-12:00	DD-QJ-TB: BD strategy meeting
12:30-18:00	DD, JPC, DBr-SAB meeting @ Hotel Château Versailles

Thursday 7
9:00-18:00	Senior Management Retreat DD QJ DBr JPC CL DCS FN TM XH NBr ML KP IP - Safety Meeting
9:00-10:00	

Friday 8
9:00-18:00	Senior Management Retreat DD QJ DBr JPC CL DCS FN

Saturday 9
<--DB travel to SFO-->

Sunday 10
<--JPC BL HD DB - Travel to SFP-->
<--JPC BL HD - Travel to SFO-->

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de. Stringent conditions may also be achieved by the addition of destabilizing agents, such as formamide. Such a nucleotide according to the invention may be of any length in accordance with the above defined.

In a preferred embodiment of the invention standard stringency conditions are 2 x SSC and high stringency conditions as 1 x SSC (see Church and Gilbert, *Proc Nat Acad Sci USA* (1984) 81, 1991-1995 incorporated herein by reference).

The nucleic acids according to the invention are cloned or amplified by *in vitro* methods, such as polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), etc. A wide variety of cloning and *in vitro* amplification methods are well known to persons of skill, see e.g. Berger and Kimmel, Guide to Molecular Cloning Techniques, *Methods in Enzymology* 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook et al., (1989) *Molecular Cloning - A Laboratory Manual*, vol 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY; Current Protocols in Molecular Biology, F.M. Ausbel et al., eds., Current Protocols; Cashion et al., US patent no. 5 017 478; and Carr, EP patent no. 0 246 864.

Another aspect of the present invention is an antibody raised against a peptide, polypeptide or protein according to the invention. Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (antigen), preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (e.g. GST, keyhole limpet hemanocyanin etc.), or a polypeptide incorporated into an immunization vector such as a recombinant vaccinia virus (see US patent no. 4 722 848) is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of the reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired (see. e.g. Coligan (1991) Current Protocols in Immunology, Wiley/Greene, NY; and Harlow and Lane (1989) Anti-

bodies: A laboratory manual, Cold Spring Harbor Press, NY). In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans etc. Description of techniques for preparing such monoclonal antibodies are found in e.g. Stites et al., (eds.) Basic and Clinical Immunology (4th ed), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, supra; Goding (1986), Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) Nature 256: 495-497.

An additional object is the use of a molecule as defined above, eg. a cDNA, in gene therapy as well as such gene therapy methods. The methods according to the invention can involve transfecting cells of a mammal with a vector expressing a polypeptide or antibody according to the invention. The transfection can be *in vivo* or ex vivo. Ex vivo transfection is suitably followed by re-infusing the cells into the organism. Other methods involve administering to the mammal, e.g. a human, of a therapeutically effective dose of a composition comprising a polypeptide according to the invention and a pharmacological excipient and/or carrier.

For a review of gene therapy procedures, see Anderson, Science (1992) 256:808-813; Nabel and Felgner (1993) TIBTECH 11: 211-217; Mitani and Caskey (1993) TIBTECH 11: 162-166; Mulligan (1993) Science 926-932; Dillon (1993) TIBTECH 11: 167-175; Miller (1992) Nature 357: 455-460; Van Brunt (1988) Biotechnology 6(10): 1149-1154; Vigne (1995) Restorative Neurology and Neuroscience 8: 35-36; Kremer and Perricaudet (1995) British Medical Bulletin 51(1) 31-44; Haddada et al. (1995) in Current Topics in Microbiology and Immunology Doerfler and B÷hm (eds) Springer-Verlag, Heidelberg Germany; and Yu et al., Gene Therapy(1994) 1:13-26.
One further object of the present invention is to provide a method of treating diseases and processes that are mediated by endothelial cell proliferation, especially angiogenesis. One such disease which may be treated is cancer.

Thus this aspect of the invention particularly includes a method of treating a patient in need of anti-angiogenic therapy comprising administering to that patient an effective dose of one or more proteins or peptides which individually or together comprise (a) an amino acid sequence corresponding to that of one or more of Kringles 1, 2 and 3 of a mammalian plasminogen or anti-angiogenically active peptide fragments or protected derivatives thereof and (b) an amino acid sequence corresponding to that of peptides and proteins selected from the group consisting of a mammalian plasminogen Kringle 5, its fusion proteins or anti-angiogenically active peptide fragments or protected derivatives thereof wherein the total molecular weight of the administered protein and/or peptides (a) + (b) is no more than 50-70kDa, preferably 50-65kDa, as defined by reducing polyacrylamide gel electrophoresis.

Preferred molecular weights are as described for the components of the compositions of the invention above. Preferred sequences correspond to the respective part of the human plasminogen sequence.

In a preferred such method the protein of amino acid sequence corresponding to one or more of K1, K2 and K3 of a mammalian plasminogen consists of a protein corresponding to the amino acid sequence of a mammalian plasminogen fragment stated to be a preferred form of the composition of the invention and is of 45KDa or less as determined by reducing polyacrylamide gel electrophoresis.

Still more preferably amino acid sequence (b) corresponding to that of a mammalian plasminogen Kringle 5, its fusion proteins or an anti-angiogenically active peptide fragment or protected derivative thereof is selected from the group of molecules comprising the amino acid sequence corresponding to that of mammalian plasminogen Kringle 5 Arg530 to Tyr534 and molecules comprising the amino acid sequence of mammalian plasminogen Kringle 5 Pro150 to Tyr 156.

Still more preferably the peptide or protein having amino acid sequence corresponding to that of a mammalian plasminogen Kringle 5, its fusion proteins or anti-

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angiogenically active peptide fragments or protected derivatives thereof is selected from the group of molecules comprising the amino acid sequence of mammalian plasminogen Kringle 5 Val454 to Ala543.

More preferably only one anti-angiogenically active protein is administered and it comprises an amino acid sequence corresponding to that of mammalian K 1, 2 or 3, more preferably K1, together with that of K 5 in the same sequence. Most preferably the K1, K2 or K3 sequence is fused directly to the K5 sequence and most preferably in the order NH2-K1-K5-CO2H, NH2-K1-2-K5-CO2H and NH2-K1-3K5-CO2H.

Conveniently the protein comprises Kringle 1, 2 or 3 and Kringle 5 joined together by a spacer amino acid sequence and more conveniently the spacer sequence corresponds to that of a mammalian Kringle 4. Most conveniently the anti-angiogenically active protein comprises an amino acid sequence corresponding to that of a mammalian plasminogen Kringles 1 to 5 Lys78 to Arg529 .

Accordingly, in one preferred aspect, the invention particularly relates to a method of treating a human or animal with an angiogenic disease comprising administering, to said human or animal, a molecule or protein having a molecular weight of approximately 50 - 65 kilodaltons as determined by non-reducing polyacrylamide gel electrophoresis and having an amino acid sequence substantially similar to that of a plasminogen fragment comprised of Lys 78-Arg 530 of a plasminogen molecule. In a preferred embodiment, the protein has a molecular weight of approximately 50 - 60 kilodaltons, preferably 53 -57 kilodalton and most preferred approximately 55 kilodalton.

The method according to the invention may use a molecule substantially similar to a plasminogen fragment, which is selected from the group consisting of human plasminogen, murine plasminogen, bovine plasminogen, Rhesus plasminogen and porcine plasminogen.

Thus, the present invention encompasses protein molecules which are analogues of kringle 1-5 of plasminogen as well as methods of use thereof. The new part which has been added to the previously known angiostatin molecule is accordingly kringle no 5 of plasminogen, which is separately described in detail below. Previous tests have shown that kringle no 5 of plasminogen possesses a certain inhibitory effect alone (see US Patent 5,854.221, filed on December 12, 1996, by the present inventor, which application was secret at the time of the filing of the priority document of the present application and which is hereby included via reference). However, the effect of K5 alone is hundreds of times weaker that the effect of the molecule according to the present invention. In addition, the endothelial cell proliferation inhibitory effect of the molecule or molecules according to the present invention is far superior than a mere addition of the effects of K1-4 and K-5 and much greater than the addition of the very weakly active K1 when administered alone.

The present invention also encompasses diagnostic and therapeutic methods for detecting the presence or absence of the inhibiting peptide in body fluids, and for administration of the peptide or antibodies that specifically bind the peptide to patients in need of therapeucically effective amounts of such compounds to regulate endothelial cell proliferation. Additionally, the inhibitory peptide may be used in conjunction with *in vitro* proliferating endothelial cell cultures to test for compounds that mitigate the inhibitory effects of the peptide - i.e. to screen for growth factors or other compounds capable of overcoming or reversing the inhibition of endothelial cell proliferation. Thus, another aspect of the invention is a screening assay, wherein a nucleic acid, a protein, a peptide or a polypeptide according to the invention is used. For a review of general immunoassays, see e.g. Methods in Cell Biology, vol. 37: Antibodies in Cell Biology, Asai, ed. Academic Press, Inc. New York (1993); Basic and Clinical Immunology 7th ed., Stites & Terr, eds., (1991). The immunoassays may be competitive or non-competitive. Thus, the present invention also encompasses screening assays aimed at the detection of molecules useful as alternatives to K1-5, which exhibits the same function but are more desirable e-g- due to a smaller molecular weight.

Thus, the methods according to the invention are aimed at the detection of peptide or polypeptides, or even proteins, that bind to the same target as K1-5 and therefore exhibit an equivalent therapeutic effect. The peptides or polypeptides, or even proteins detected by the method of the invention are also within the scope of the invention and thus, a further aspect of the invention is any peptide, polypeptide, or protein that acts in the same manner or binds to the same receptors as K1-5 as defined by the invention, preferably by SEQ ID NO. 1 and SEQ ID NO 2. Such same manner particularly means that it acts via two or more anti-angiogenic mechanisms such as to have an enhanced efficacy at least equal to that of K1-5 weight by weight of dose administered.

Still another object of the present invention is to provide a diagnostic or prognostic method, or assay, and kit for detecting the presence and/or amount of the inhibitor in biological fluid samples, such as a body fluid or tissue. In addition, the invention also relates to to histochemical kits for localization of the inhibitor defined herein.

Another object of the invention is to provide molecular probes to monitor inhibitor biosynthesis and degradation, antibodies that are specific for the inhibitor according to the invention, the development of peptide agonists and antagonists to said inhibitor's receptor, anti-inhibitor receptor-specific antibody agonists and antagonists, and to cytotoxic agents linked to the inhibitor.

It is yet another object of the present invention to provide methods and compositions for treating diseases and processes that are mediated by angiogenesis including, but not limited to, hemangioma, solid tumors, leukemia, metastasis, telangiectasia, psoriasis, scleroderma, pyogenic granuloma, myocardial angiogenesis, plaque neovascularization, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy, retrolental fibroplasia, arthritis, diabetic neovascularization, macular degeneration, wound healing, peptic ulcer, Helicobacter related diseases, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, placentation, and cat scratch fever.

It is another object or the present invention to provide a composition for treating or repressing the growth of a cancer.

It is an object of the present invention to provide compounds that modulate or mimic the production or activity of enzymes that produce the inhibitor of the present invention *in vivo* or *in vitro*.

It is a further object of the present invention to provide the inhibitor or anti-inhibitor antibodies by direct injection of inhibitor DNA into a human or animal needing such treatment.

It is an object of present invention to provide a method for detecting and quantifying the presence of an antibody specific for the inhibitor in a body fluid.

It is another object of the present invention to provide a method for the detection or prognosis of cancer.

It is another object of the present invention to provide a composition for use in visualizing and quantitating sites of inhibitor binding *in vivo* and *in vitro*.

It is yet another object of the present invention to provide a composition for use in detection and quantification of inhibitor biosynthesis.

It is yet another object of the present invention to provide a therapy for cancer that has minimal side effects, such as gene therapy utilizing a molecule as defined above.

Still another object of the present invention is to provide a composition comprising the endothelial cell proliferation inhibitor of the present invention or inhibitor peptide fragment linked to a cytotoxic agent.

Another object of the present invention is to provide a method for targeted delivery of inhibitor-related compositions to specific locations.

Yet another object of the invention is to provide compositions and methods useful for gene therapy for the modulation of endothelial cell proliferation, such as angiogenic processes.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

Detailed description of the drawings

Fig. 1 Proteolytic human K1-5 fragment

(A) Kringle 1-5 fragment of human plasminogen was obtained by digestion of Glu1 plasminogen with urokinase-activated plasmin. The cleavage sites of plasmin is located between the double positively charged Lys76 (77) and Lys77 (78) at the N-terminus, and Arg529 (530) and Lys530 (531) and the C-terminus. The K1-5 fragment containing the region between Lys77-Arg529 was purified by lysine-Sepharose chromatography followed by a Sephadex G-75 column. (B) the purified K1-5 was analyzed on a 10-15% SDS gradient polyacrylamide gel. The molecular standards in kilodaltons are indicated on the left.

Fig. 2. Anti-endothelial cell proliferative activity and Inhibition of capillary endothelial cell proliferation

K1-5 purified from the plasmin digestion of human plasminogen was assayed on bovine cappilary endothelial (BCE) cells in the presence of 1 ng/ml bFGF in a 72-h proliferation assay as described previously (Cao et al., J. Biol. Chem 271,29461-29467, 1996; Cao et al., J. Biol. Chem. 272, in press, 1997). The inhibitory activity of K1-5 on BSE cells was tested at low concentrations (0.2-10 nM) and (B) at high concentration (20 nM-320 nM).

(C) The half maximal inhibition of K1-5 on BCE cells was observed at approximately 50 pM. (D) The inhibitory effect is reversible. After removal of K1-5 from the conditioned medium, endothelial cells are able to proliferate in fresh DMEM medium containing 1 ng/ml of bFGF.

Fig. 3 Morphology of K1-5-treated endothelial cells

In the presence of various concentrations of K1-5, BCE cells stopped proliferation (B-D) as compared to control cells (A). These data suggest that K1-5 even at high concentrations is not toxic to endothelial cells.

Fig. 4 Synergistic suppression of endopthelial cell growth by angiostatin and K5

Pure human angiostatin (K1-4) and Kringle 5 (K5) were obtained as proteolytic fragments. These two endothelial cell inhibitors were added either separately or together to bovine capillary endothelial (BCE) cells stimulated by 1ng/ml basic fibroblast growth factor (bFGF). At the concentration of 1 nM, angiostatin did not exhibit endothelial cell inhibition. Kringle 5 inhibited endothelial cell proliferation by approximately 50%. When angiostatin and K5 were added together to BCE cells, approximately 95% suppression of cell growth was detected, indicating that both fragments synergistically inhibit endothelial cell proliferation.

Fig. 5. Antiangiogenic effect of K1-5 on the chick embryo chorioallantoic membrane (CAM)

Methylcellulose disk containing various doses of K1-5 was implanted on CAMs of day 6-old-embryos as described (Cao et al., J. Exp. Med. 182, 2069-2077, 1995). The number of CAMs with avascular zones over the total number of CAMs is indicated as percentages. Phosphate-buffered saline was used as a negative control.

Fig. 6. Suppression of primary tumor growth by K1-5

Approximately, 1×10^6 murine T241 fibrosarcoma tumor murine T241 fibrosarcoma tumor cells were subcutaneously implanted into each 6-week old C57B16/J mouse. The mice were systemically treated with 100μl PBS or 2.5 mg/kg/day K1-5 in 100μl PBS once daily. (A) K1-5-treated mice and a control mouse were photographed at day 20 of treatment. (B). Tumor volumes were measured with a digital calipper and calculated according to a standard formula with x with x length x 0.52. The data represent average mean values (+SEM).

Fig. 7. Detection of microvessel density by immunohistochemical staining of tumor tissue sections with anti-vWF antibodies

(A) A control tumor section treated with PBS. (B) and (C) K1-5-treated tumor sections. (D) Quantification of microvessels of tumor tissue sections per high field.

Figure 8: Synergistic suppression of endopthelial cell growth by K1 and K5 and K1-3 and K5

Pure human Kringle 1, Kringle 1-3 and Kringle 5 (K5) were obtained as proteolytic fragments. These endothelial cell inhibitors were added either separately or together to bovine capillary endothelial (BCE) cells stimulated by 1ng/ml basic fibroblast growth factor (bFGF). At the concentration of 1 nM, K1 did not exhibit any significant endothelial cell inhibition. Kringle 1-3 inhibited endothelial cell proliferation by approximately 30% and Kringle 5 by 50%. Use of Kringle 1 or Kringle 1-3 together with Kringle 5 in each case gave at least 100% inhibition of FGF-2 stimulated angiogenesis and results suggest inhibition basal level angiogenesis.

Advantageous uses of the invention

In accordance with the present invention, compositions and methods are provided that are effective for inhibiting endothelial cell proliferation, modulating angiogenesis, and inhibiting unwanted angiogenesis, especially angiogenesis related to tumor growth. The present invention includes e.g. compositions comprising an approximately 452 amino acid sequence derivable from human plasminogen as kringles 1-5. The amino acid sequence of the inhibitor may vary slightly between species.

It is to be understood that the number of amino acids in the active inhibitor molecule may vary and that all closely homologous amino acid sequences that have endothelial inhibiting activity are contemplated as being included in the present invention.

The present invention particularly provides methods and compositions for treating diseases and processes mediated by undesired and uncontrolled epithelial cell proliferation, such as angiogenesis, by administering to a human or animal having undesired

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endothelial cell proliferation a composition comprising approximately kringle 1-5 of human plasminogen capable of inhibiting endothelial cell proliferation in *in vitro* assays. Desirably, the isolated protein is at least approximately 80% pure, more desirably at least approximately 90% pure and even more desirable at least approximately 95% pure. The present invention is particularly useful for treating, or for repressing the growth of, tumors. Administration of the inhibitor to a human or animal with prevascularized metastasized tumors helps prevent the growth or expansion of those tumors.

The present invention also encompasses DNA sequences encoding the endothelial cell proliferation inhibitors, expression vectors containing DNA sequences encoding the endothelial cell proliferation inhibitors eg such as viral vectors, and cells containing one or more expression vectors containing DNA sequences encoding the inhibitors.

The present invention further encompasses gene therapy methods whereby DNA sequences encoding the endothelial cell proliferation inhibitors are introduced into a patient to modify *in vivo* inhibitor levels. Such a single sequence or a single vector comprising one or more sequences may encode the separate angiostatin like and K5 like activity mediating agents or the fusion protein or peptides described above for use in the composition of the invention.

The present invention also includes diagnostic methods and kits for detection and measurement of the endothelial cell proliferation inhibitor in biological fluids and tissues, and for localization of the inhibitor in tissues and cells.The diagnostic method and kit can be in any configuration well known to those of ordinary skill in the art. The present invention also includes antibodies specific for the endothelial cell proliferation inhibitor and portions thereof, and antibodies that inhibit the binding of antibodies specific for the endothelial cell proliferation inhibitor. These antibodies can be polyclonal antibodies or monoclonal antibodies. The antibodies specific for the endothelial cell proliferation inhibitor can be used in diagnostic kits to detect the presence and quantity of the inhibitor which is diagnostic or prognostic for the occurrence or recurrence of cancer or other disease mediated by angiogenesis. Antibodies specific for the endothelial

cell proliferation inhibitor may also be administered to a human or animal to passively immunize said human or animal against the inhibitor, thereby reducing angiogenic inhibition.

The present invention also includes diagnostic methods and kits for detecting the presence and quantity of antibodies that bind the endothelial cell proliferation inhibitor in body fluids. The diagnostic method and kit can be in any configuration well known to those of ordinary skill in the art.
The present invention also includes anti-inhibitor receptor-specific antibodies that bind to the inhibitor's receptor and transmit the appropriate signal to the cell and act as agonists or antagonists.

The present invention also includes inhibitor peptide fragments and analogs that can be labeled isotopically or with other molecules or proteins for use in the detection and visualization of the inhibitor binding sites with techniques, including, but not limited to, positron emission tomography, autoradiography, flow cytometry, radioreceptor binding assays, and immunohistochemistry.

These inhibitor peptides and analogs also act as agonists and antagonists at the inhibitor receptor, thereby enhancing or blocking the biological activity of the endothelial cell proliferation inhibitor. Such peptides are used in the isolation of the receptor molecules capable or specifically binding to the inhibitor.

The present invention also includes the endothelial cell proliferation inhibitor, inhibitor fragments, antisera specific for the inhibitor, and inhibitor receptor agonists and receptor antagonists linked to cytotoxic agents for therapeutic and research applications.

Still further, the inhibitors, fragments thereof, antisera specific therefore, inhibitor receptor agonists and inhibitor receptor antagonists are combined with pharmaceutically acceptable excipients, and optionally sustained-release compounds or compositions, such as biodegradable polymers and matrices, to form therapeutic compositions.

The present invention includes molecular probes for the ribonucleic acid and deoxyribonucleic acid involved in transcription and translation of the endothelial cell proliferation inhibitor. These molecular probes are useful for detecting and measuring inhibitor biosynthesis in tissues and cells.

The inhibitors may be isolated from plasminogens, such as human plasminogen, or synthesized by chemical or biological methods (e.g. cell culture, recombinant gene expression, peptide synthesis and *in vitro* enzymatic catalysis of plasminogen or plasmin to yield active inhibitor). Recombinant techniques include gene amplification from DNA sources using the polymerase chain reaction (PCR), and gene amplification from RNA sources using reverse transcriptase/PCR.

Suitable general methods for obtaining kringles are described in Cao et al (1996) referenced above incorporated herein by reference.

The present invention also encompasses a composition comprising a vector containing a DNA sequence encoding the endothelial cell proliferation inhibitor, wherein the vector is capable of expressing the inhibitor or synergistic inhibitors when present in a cell, a composition comprising a cell containing a vector, wherein the vector contains a DNA sequence encoding the inhibitor fragments or analogs thereof, and wherein the vector is capable of expressing the inhibitor when present in the cell, and a method comprising implanting into a human or non-human animal a cell containing a vector, wherein the vector contains a DNA sequence encoding the inhibitor, wherein the vector is capable of expressing the inhibitor when present in the cell.

As used herein, the term "substantially similar" or "substantially homologous" when used in reference to the inhibitor amino acid and nucleic acid sequences, means an amino acid sequence having endothelial cell proliferation inhibiting activity and and having a similar molecular weight eg. of approximately 55kDa for K1-5, which also has a high degree of sequence homology to the protein having the specific N-terminal amino acid sequence disclosed herein, or a nucleic acid sequence that codes for an en-

dothelial cell proliferation inhibitor having a molecular weight of approximately 55kDa and a high degree of homology to the having the specific N-terminal amino acid sequence disclosed herein.

A high degree of homology means at least approximately 80% amino acid homology, desirably at least approximately 90% amino acid homology, and more desirably at least approximately 95% amino acid homology; more preferably having such percentages of identity. The term "endothelial inhibiting activity" as used herein means the capabilitv of a molecule to inhibit angiogenesis in general and, for example, to inhibit the growth of bovine capillary endothelial cells in culture in the presence of fibroblast growth factor.

Such homology can be readily determined by use of one of the commercially or otherwise publicly available software packages. Algorithms and software suitable for use in aligning sequences for comparison and calculation of sequence homology or identity will be known to those skilled in the art. Significant examples of such tools are the Pearson and Lipman search based FAST and BLAST programs. Details of these may be found in Altschul et al (1990), J. Mol. Biol. 215: 403-10; Lipman D J and Pearson W R (1985) Science 227, p1435-41. Publically available details of BLAST may be found on the internet at æhttp://www.ncbi. nlm.nih.gov/BLAST/blast-help.htmlÆ. Thus such homology and identity percentages can be ascertained using commercially or publicly available software packages incorporating, for example, FASTA and BLASTn software or by computer servers on the internet. An example of the former is the GCG Wisconsin   Software   package   while   both   Genbank   (see http://www.ncbi.nlm.nih.gov/BLAST)   and   EMBL: (see   http://www.embl-heidelberg.de/Blast2) offer internet services. Default settings are conveniently used.

When the homology between the human and mouse sequence of K1-5 according to the invention was determined using BLAST 2.0.6, a homology of 88% was found.

By the term identity is meant that the stated percentage of the claimed amino acid se-
quence or base sequence is to be found in the reference sequence in the same relative
positions when the sequences are optimally aligned, notwithstanding the fact that the
sequences may have deletions or additions in certain positions requiring introduction
of gaps to allow alignment of the highest percentage of amino acids or bases. Prefe-
rably the sequence are aligned by using 10 or less gaps, ie. the total number of gaps
introduced into the two sequences when added together is 10 or less. The length of
such gaps is not of particular importance as long as the anti-angiogenic activity is re-
tained but generally will be no more than 10, and preferably no more than 5 amino
acids, or 30 and preferably no more than 15 bases.

Preferred parameters for BLAST searches are the default values, ie. for EMBL Advan-
ced Blast2: Blastp Matrix BLOSUMS, Filter default, Echofilter X, Expect 10, Cutoff
default, Strand both, Descriptions 50, Alignments 50. For BLASTn defaults are again
preferably used. GCG Wisconsin Package defaults are Gap Weight 12, Length weight
4. FASTDB parameters used for a further preferred method of homology calaculation
are mismatch penalty = 1.00, gap penalty =1.00, gap size penalty = 0.33 and joining
penalty =30.0.

When the identity between the human and mouse sequence of K1-5 according to the
invention was determined using BLAST 2.0.6, an identity of 77% was found.

The present invention also includes the detection of the inhibitor or inhibitors in body
fluids and tissues for the purpose of diagnosis or prognosis of diseases such as cancer.
The present invention also includes the detection of inhibitor binding sites and recep-
tors in cells and tissues. The present invention also includes methods of treating or
preventing angiogenic diseases and processes including, but not limited to, arthritis and
tumors by stimulating the production of the inhibitor, and/or by administering isolated
inhibitor, or desirable purified inhibitor, or inhibitor agonists or antagonists, and/or
inhibitor-specific antisera or antisera directed against inhibitor-specific antisera to a
patient. Additional treatment methods include administration of the inhibitor, biologi-

cally active fragments thereof, inhibitor analogs, inhibitor-specific antisera, or inhibitor receptor agonists and antagonists linked to cytotoxic agents.

Passive antibody therapy using antibodies that specifically bind the inhibitor can be employed to modulate angiogenic-dependent processes such as reproduction; development, and wound healing and tissue repair. In addition, antisera directed to the Fab regions of inhibitor-specific antibodies can be administered to block the ability of endogenous inhibitor-specific antisera to bind inhibitor.

The present invention also encompasses gene therapy, whereby the gene encoding the inhibitor or inhibitors is regulated in a patient. Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise rereferred to as gene therapy, are disclosed in Gene Transfer into Mammalian Somatic Cells *in vivo*, N. Yang,Crit. Rev. Biotechn.12 (4): 335-356 (1992), which is hereby incorporated by reference. Gene therapy encompasses incorporation of DNA sequences into somatic cells or germ line cells for use in either ex vivo or *in vivo* therapy.Gene therapy functions to replace genes, augment normal or abnormal gene function, and to combat infectious diseases and other pathologies.

Strategies for treating these medical problems with gene therapy include therapeutic strategies, such as identifying the defective gene and then adding a functional gene to either replace the function of the defective gene or to augment a slightly functional gene; or prophylactic strategies, such as adding a gene to the product protein that will treat the condition or that will make the tissue or organ more susceptible to a treatment regimen. As an example of a prophylactic strategy, a nucleic acid sequence coding for the inhibitor may be placed in a patient and thus prevent occurrence of angiognesis; or a gene that makes tumor cells more susceptible to radiation could be inserted and then radiation of the tumor would cause increased killing of the tumor cells.

Many protocols for transfer of inhibitor DNA or inhibitor reaulatory sequences are envisioned in this invention. Transfection of promoter sequences, other than one nor-

mally found specifically associated with the inhibitor, or other sequences which would increase production of the inhibitor protein are also envisioned as methods of gene therapy. An example of this technology is found in Transkaryotic Therapies, Inc., of Cambridge, Massachusetts, using homologous recombination to insert a "genetic switch" that turns on an erytropoietin gene in cells. See Genetic Engineering News, April 15, 1994. Such "genetic switches" could be used to activate the inhibitor (or the inhibitor receptor) in cells not normally expressing the inhibitor (or the receptor for the inhibitor).

Gene transfer methods for gene therapy fall into three broad categories - physical (i.e. electroporation, direct gene transfer and particle bombardment), chemical (lipid-based carriers, or other non-viral vectors) and biological (virus-derived vector and receptor uptake). For example, non-viral vectors may be used which include liposomes coated with DNA. Such liposome/DNA complexes may be directly injected intravenously into the patient. It is believed that the liposome/DNA complexes are concentrated in the liver where they deliver the DNA to macrophages and Kupfter cells. These cells are long lived and thus provide long term expression of the delivered DNA. Additionally, vectors or the "naked" DNA of the gene may be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes include ex vivo gene transfer, *in vivo* gene transfer and *in vitro* gene transfer. In ex vivo gene transfer, cells are taken from the patient and grown in cell culture. The DNA is transfected into the cells, the transfected cells are expanded in number and then reimplanted in the patient. In *in vitro* gene transfer, the transformed cells are cells growing in culture, such as tissue culture cells, and not particular cells from a particular patient. These "laboratory cells" are transfected, the transfected cells are selected and expanded for either implantation into a patient or for other uses.

In vivo gene transfer involves introducing the DNA into the cells of the patient when the cells are within the patient. Methods include using a noninfectous virus to deliver

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the gene in the patient or injecting naked DNA into a site in the patient and the DNA is taken up by a percentage of cells in which the gene product protein is expressed. Additionally, the other methods described herein, such as use of a "gene gun", may be used for *in vitro* insertion ot endothelial cell proliferation inhibitor DNA or inhibitor regulatory sequences.

Chemical methods of gene therapy may involve a lipid based compound, not necessarily a liposome, to ferry the DNA across the cell membrane. Lipofectins or cytofectins, lipid-based positive ions that bind to negatively charged DNA, make a complex that can cross the cell membrane and provide the DNA into the interior of the cell. Another chemical method uses receptor-based endocytosis, which involves binding a specific ligand to a cell surface receptor and developing and transporting it across the cell membrane. The ligand binds to the DNA and the whole complex is transported into the cell. The ligand gene complex is injected into the blood stream and then target cells that have the receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

Many gene therapy methodologies employ viral vectors to insert genes into cells. For example, altered retrovirus vectors have been used in ex vivo methods to introduce genes into peripheral and tumor-infiltrating lymphocytes, hepatocytes, epidermal cells, myocytes, or other somatic cclls. These altered cells are then introduced into the patient to provide the gene product from the inserted DNA. Viral vectors have also been used to insert genes into cells using *in vivo* protocols. To direct tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue specific can be used. Alternatively, this can be achieved using in situ delivery of DNA or viral vectors to specific anatomical sites *in vivo*. For example, gene transfer to blood vessels *in vivo* was achieved by implanting *in vitro* transduced endothelial cells in chosen sites on arterial walls. The virus infected surrounding cells which also expressed the gene product. A viral vector can be delivered directly to the *in vivo* site, by a catheter for example, thus allowing only certain areas to be infected by the virus, and providing long-term, site specific gene expression. *In vivo* gene transfer using retrovi-

rus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Viral vectors that have been used for gene therapy protocols include but are not limited to, retroviruses, other RNA viruses such as polio virus or Sindbis virus, adenovirus, adeno-associated virus, herpes viruses, SV 40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors are the most widely utilized gene transfer vectors. Murine leukemia retroviruses are composed of a single strand RNA complexed with a nuclear core protein and polymerase (pol) enzymes, encased by a protein core (gag) and surrounded by a glycoprotein envelope (env) that determines host range. The genomic structure of retroviruses include the gag, pol and env genes enclosed at by the 5' and 3' long terminal repeats (LTR). Retroviral vector systems exploit the fact that a minimal vector containing the 5' and 3' LTRs and the packaging signal are sufficient to allow vector packaging, infection, and integration into target cells providing that the viral structural proteins are supplied in trans in the packaging cell line. Fundamental advantages of retroviral vectors for gene transfer include efficient infection and gene expression in most cell types, precise single copy vector into target cell chromosomal DNA, and ease of manipulation of the retroviral genome.

The adenovirus is composed of linear double stranded DNA complexed with core proteins and surrounded with capsid proteins. Advances in molecular virology have led to the ability to exploit the biology of these organisms in order to create vectors capable of transducing novel genetic sequences into target cells *in vivo*. Adenoviral-based vectors will express gene product peptides at high levels. Adenoviral vectors have high efficiencies of infectivity, even with low titers of virus. Additionally, the virus is fully infective as a cell free virion so injection of producer cell lines are not necessary. Another potential advantage to adenoviral vectors is the ability to achieve long term expression of heterologous genes *in vivo*.

Mechanical methods of DNA delivery include fusogenic lipid vesicles such as liposomes or other vesicles for membrane fusion, lipid particles of DNA incorporating catio-

nic lipid such as lipofectin, polylysine-mediated transfer of DNA, direct injection of DNA, such as microinjection of DNA into germ or somatic cells, pneumatically delivered DNA-coated particles, such as the gold particles used in a "gene gun", and inorganic chemical approaches such as calcium phosphate transfection. Another method, ligand mediated gene therapy, involves complexing the DNA with specific ligands to form ligand-DNA conjugates to direct the DNA to specific cells or tissue.

It has been found that injecting plasmid DNA into muscle cells yields high percentage of the cells which are transfected and have sustained expression of marker genes. The DNA of the plasmid may or may not integrate into the genome ot the cells. Non-integration of the transfected DNA would allow the transfection and expression of gene product proteins in terminally differentiated, non-proliferative tissues for a prolonged period of time without fear of mutational insertions, deletions, or alterations in the cellular or mitochondrial genome. Long-term, but not necessarily permanent transfer of therapeutic genes into specific cells may provide treatments for genetic diseases or for prophylactic use. The DNA could be reinjected periodically to maintain the gene product level without mutations occuring in the genomes of the recipient cells. Non-integration of exogenous DNAs may allow for the presence of several different exogenous DNA constructs within one cell with all of the constructs expressing various gene products.

Particle-mediated gene transfer methods were first used in transforming plant tissue. With a particle bombardment device, or "gene gun", a motive force is generated to accelerate DNA-coated high density particles (such as gold or tungsten) to a high velocity that allows penetration of the target organs, tissues or cells. Particle bombardment can be used in *in vitro* systems, or with ex vivo or *in vivo* techniques to introduce DNA into cells, tissues or organs.

Electroporation for gene transfer uses an electrical current to make cells or tissues susceptible to electroporation-mediated gene transfer. A brief electric impulse with a given field strength is used to increase the permeability of a membrane in such a way that DNA molecules can penetrate into the cells. This technique can be used in *in vitro*

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systems, or with ex vivo or *in vivo* techniques to introduce DNA into cells, tissues or organs.

Carrier mediated gene transfer *in vivo* can be used to transfect foreign DNA into cells. The carrier-DNA-complex can be conveniently introduced into body fluids or the bloodstream and then site specifically directed to the target organ or tissue in the body. Both liposomes and polycations, such as polylysine, lipofectins or cytofectins can be used. Liposomes can be developed which are cell specific or organ specific and thus the foreign DNA carried by the liposome will be taken up by target cells. Injection of immunoliposomes that are targeted to a specific receptor on certain cells can be used as a convenient method of inserting the DNA into the cells bearing the receptor. Another carrier system that has been used is the asialoglycoportein/polylysine conjugate system for carrying DNA to hepatocytes for *in vivo* gene transfer.

The transfected DNA may also be complexed with other kinds of carriers so that the DNA is carried to the recipient cell and then resides in the cytoplasm or in the nucieoplasm. DNA can be coupled to carrier nuclear proteins in specifically engineered vesicle complexes and carried directly into the nucleus.

Gene regulation of the inhibitor or inhibitors of the present invention may be accomplished by administering compounds that bind to the gene for the inhibitor, or control regions associated with the gene, or its corresponding RNA transcript to modify the rate of transcription or translation. Additionally, cells transfected with a DNA sequence encoding the inhibitor may be administered to a patient to provide an *in vivo* source of inhibitor. For example, cells may be transfected with a vector containing a nucleic acid sequence encoding the inhibitor.

The term "vector" as used herein means a carrier that can contain or associate with specific nucleic acid seqnences, which functions to transport the specific nuclic acid sequences into a cell. Examples of vectors include plasmids and infective microorganisms such as viruses, or non-viral vectors such as ligand-DNA conjugates, liposomes,

lipid- DNA complexes. It may be desirable that a recombinant DNA molecule comprising an endothelial cell proliferation inhibitor DNA sequence is operatively linked to an expression control sequence to form an expression vector capable of expressing the inhibitor. The transfected cells may be cells derived from the patient's normal tissue, the patient's diseased tissue, or may be non-patient cells.

For example, tumor cells removed from a patient can be transfected with a vector capable of expressing the inhibitor protein of the present invention and re-introduced into the patient. The transfected tumor cells produce levels of inhibitor in the patient that inhibit the growth of the tumor. Patients may be human or non-human animals. Additionally, inhibitor DNA may be directly injected, without the aid of a carrier, into a patient. In particular, inhibitor DNA may be injected into skin, muscle or blood.

Inhibitor expression may continue for a long-period of time or inhibitor DNA may be administered periodically to maintain a desired level of the inhibitor protein in the cell, the tissue or organ or biological fluid.

Although not wanting to be bound by the following hypothesis, it is believed that when a tumor becomes angiogenic it releases one or more angiogenic peptides (e.g. aFGF, bFGF, VEGF, IL-8, GM-CSF, etc.), which act locally, target endothelium in the neighborhood of a primary tumor from an extravascular direction, and do not circulate (or circulate with a short half-life). These angiogenic peptides must be produced in an amount sufficient to overcome the action of endothelial cell inhibitor (inhibitors of angiogenesis) for a primary tumor to continue to expand its population. Once such a primary tumor is growing well, it continues to release endothelial cell inhibitors into the circulation. According to this hypothesis, these inhibitors act remotely at a distance from the primary tumor, target capillary endothelium of a metastasis from an intravascular direction, and continue to circulate. Thus, just at the time when a remote metastasis might begin to initiate angiogenesis, the capillary endothelium in its neighborhood could be inhibited by incoming inhibitor.

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Production of the approximately endothelial cell proliferation inhibitor of the present invention in accomplished using similar techniques can be accomplished using recombinant DNA techniques including the steps of (1) identifying and purifying the inhibitor as described herein and exemplified by the Figures. (2) determining the N-terminal amino acid sequence of the purified inhibitor, (3) synthetically generating 5' and 3' DNA oligonucleotide primers for the inhibitor sequence, (4) amplifying the inhibitor gene sequence using polymerase, (5) inserting the amplfied sequence into an appropriate vector such as an expression vector, (6) inserting the gene containing vector into a microorganism or other expression system capable of expressing the inhibitor gene, and (7) isolating the recombinantly produced inhibitor. Appropriate vectors include viral, bacterial and eukaryotic (such as yeast) expression vectors. The above techniques are more fully described in laboratory manuals such as "Molecular Cloning: A Laboratory Manual" Second Edition by Sambrook et al., Cold Spring Harbor Press, 1989, which is incorporated herein by reference. The contents of all references cited in this application are included herein by reference.

Yet another method of producing the inhibitor, inhibitors or biologically active fragments thereof, is by peptide synthesis. The amino acid sequence of the inhibitor can be determined, for example by automated peptide sequencing methods. Alternatively, once the gene or DNA sequence which codes for inhibitor or inhibitors is isolated, for example by the methods described above, the DNA sequence can be determined using manual or automated sequencing methods well know in the art. The nucleic acid sequence in turn provides information regarding the amino acid sequence.

Once the amino acid sequence of the peptide is known, peptide fragments can be synthesized by techniques well known in the art, as exemplified bv "Solid Phase Peptide Synthesis: A Practical Approach" E. Atherton and R.C. Sheppard. IRL Press, Oxford, England. Multiple fragments can be synthesized which are subsequently linked together to form larger fragments. These synthetic peptide fragments can also be made with amino acid substitutions at specific locations in order to test for agonistic and antagonistic activity *in vitro* and *in vivo*. Peptide fragments that possess high affinity bin-

ding to tissues can be used to isolate receptors the bind the inhibitor on affinity columns.

The inhibitor or inhibitors are effective in treating diseases or processes such as angiogenesis, that are mediated by, or involve, endothelial cell proliferation. The present invention includes the method of treating an angiogenesis mediated disease with an effective amount of inhibitor, or a biologically active fragment thereof, or combinations of inhibitor fragments that collectively possess anti-angiogenic activity or inhibitor agonists and antagonists. The angiogenesis mediated diseases include, but are not limited to, solid tumors; blood born tumors such as leukemias; tumor metastasis; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation.

The inhibitor is useful in the treatment of diseases of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. The inhibitor can be used as a birth control agent by preventing vascularization required for embryo implantation. The inhibitor is useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (Rochele minalia quintosa) and ulcers (Helicobacter pylori).

The synthetic peptide fragments of the inhibitor have a variety of uses. The peptide that binds to receptor capable of binding the inhibitor with high specificity and avidity is radiolabeled and employed for visualization and quantitation of binding sites using autoradiographic and membrane binding techniques.

In addition, labeling inhibitor or peptide fragments thereof with short lived isotopes enables visualization of receptor binding sites *in vivo* using positron emission tomo-

graphy or other modern radiographic techniques in order to locate tumors with inhibitor binding sites.

Cytotoxic agents, such as ricin, are linked to the inhibitor, and high affinity peptide fragments thereof, thereby providing a tool for destruction of cells that bind the inhibitor. These cells may be found in many locations, including but not limited to, micrometastases and primary tumors. Peptides linked to cytotoxic agents are infused in a manner designed to maximize delivery to the desired location. For example, delivery may be accomplished through a cannula into vessels supplying the target site or directly into the target. Such agents are also delivered in a controlled manner through osmotic pumps coupled to infusion cannulae. A combination of inhibitor antagonists may be co-applied with stimulators of angiogenesis to increase vascularization of tissue. This therapeutic regimen provides an effective means of destroying metastatic cancer.

The inhibitor or inhibitors may be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy combined with the inhibitor and then the inhibitor may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor. Additionally, the inhibitor, fragments thereof, inhibitor-specific antisera, inhibitor receptor agonists and antagonists, or combinations thereof, are combined with pharmaceutically acceptable excipients, and optionally sustained-release matrix, such as biodegradable polymers, to form therapeutic compositions.

A sustained-release matrix, as used herein, is a matrix made of materials, usually polymers, which are degradable by enzymatic or acid/base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. The sustained-release matrix desirably is chosen from biocompatible materials, such as liposomes, polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (co-polymers of lactic acid and glycolic acid), polyanhydrides,

poly(ortho)esters, polypeptides, hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene, polyvinylpyrrolidone and silicone. A preferred biodegradable matrix is a matrix of one of either polylactide, polyglycolide, or polylactide co-glycolide (copolymers of lactic acid and glycolic acid).

The angiogenesis-modulating therapeutic composition of the present invention may be a solid, liquid or aerosol and may be administered by any known route of administration. Examples of solid therapeutic compositions include pills, creams, and implantable dosage units. The pills may be administered orally, the therapeutic creams may be administered topically. The implantable dosage units may be administered locally, for example at a tumor site, or which may be implanted for systemic release of the therapeutic angiogenesis modulating composition, for example subcutaneously. Examples of liquid composition include formulations adapted for injection subcutaneously, intravenously, intraarterially and formulations for topical and intraocular administration. Examples of aerosol formulations include inhaler formulation for administration to the lungs.

The inhibitor proteins and peptides of the present invention also can be used to generate antibodies that are specific for the inhibitor and its receptor. The antibodies can be either polyclonal antibodies or monoclonal antibodies. These antibodies that specifically bind to the inhibitor or inhibitor receptors can be used in diagnostic methods and kits that are well known to those of ordinary skill in the art to detect or quantify the inhibitor levels or inhibitor receptors levels in a body fluid or tissue. Results from these tests can be used to diagnose or predict the occurrence or recurrence of a cancer and other angiogenic mediated diseases.

The inhibitor or inhibitors also can be used to develop a diagnostic method and kit to detect and quantify antibodies capable of binding the inhibitor. These kits would permit detection of circulating inhibitor-specific antibodies. Patients that have such circulating anti-inhibitor antibodies may be more likely to develop multiple tumors and can-

cers, and may be more likely to have recurrences of cancer after treatments or periods of remission. The Fab fragments of these antibodies may be used as antigens to generate anti-inhibitor-specific Fab-fragment antisera which can be used to neutralize anti-inhibitor antibodies. Such a method would reduce the removal of circulating inhibitor by anti-inhibitor antibodies, thereby effectively elevating circulating inhibitor levels.

Another aspect of the present invention is a method of blocking the action of excess endogenous inhibitor. This can be done by passively immunizing a human or animal with antibodies specific for the undesired inhibitor in the system. This treatment can be important in treating abnormal ovulation, menstruation and placentation, and vasculogenesis. This provides a useful tool to examine the effects of inhibitor removal on metastatic processes. The Fab fragment of inhibitor-specific antibodies contains the binding site for inhibitor. This fragment is isolated from inhibitor-specific antibodies usig techniques known to those skilled in the art. The Fab fragment of inhibitor-specific antisera are used as antigens to generate production of anti-Fab fragment serum. Infusion of this antiserum against the Fab fragments specific for the inhibitor prevents the inhibitor from binding to inhibitor antibodies. Therapeutic benefit is obtained by neutralizing endogenous anti-inhibitor antibodies by blocking the binding of inhibitor to the Fab fragments or anti-inhibitor. The net effect of this treatment is to facilitate the ability of endogenous circulating inhibitor to reach target cells, thereby decreasing the spread of metastases.

It is to be understood that the present invention is contemplated to include any derivatives of the inhibitor that have endothelial cell proliferation inhibitor activity. The present invention includes the entire inhibitor protein, derivatives of the inhibitor protein and biologically-active fragments of the inhibitor protein. These include proteins with inhibitor activity that have amino acid substitutions or have sugars or other molecules attached to amino acid functional groups. The present invention also includes genes that code for the inhibitor and the inhibitor receptor, and to proteins that are expressed by those genes.

The proteins and protein fragments with the inhibitor activity described above can be provided as isolated and substantially purified proteins and protein fragments in pharmaceutically acceptable formulations using formulation methods known to those of oridinary skill in the art. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g., intravenous, intraspinal. subcutaneous or intramuscular) route. In addition, the inhibitor may be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the inhibitor is slowly released systemically. Osmotic minipumps may also be used to provide controlled delivery of high concentrations of the inhibitor through cannulae to the site of interest, such as directly into a metastatic growth or into the vascular supply to that tumor. The biodegradable polymers and their use are described, for example, in detail in Brem et al., J. Neurosurg. 74:441-446 (1991), which is hereby incorporated by reference in its entirety.

The dosage of the inhibitor of the present invention will depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating humans or animals, approximately 5 mg/kg/day, administered once a day, sppresses tumor growth to (50%. In the same dosages, angiostatin does not have any effect. Depending upon the half-life of the inhibitor in the particular animal or human, the inhibitor can be administered between several times per day to once a week. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

The inhibitor formulations include those suitable for oral, rectal, ophthalmic (including intravitreal or intracameral, nasal, topical (including buccal and sublingual), intrauterine, vaginal or parenteral (including subcutaneous, intraperitoneal, intramuscular, intra-

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venous, intradermal, intracranial, intratracheal, and epidural) administration. The inhibitor formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described. Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the present invention may include other agents conventional in the art having regard to the type of formulation in question. Optionally, cytotoxic agents may be incorporated or otherwise combined with inhibitor proteins, or biologically functional peptide fragments thereof, to provide dual therapy to the patient.

Angiogenesis inhibiting peptides of the present invention can be synthesized in a standard microchemical facility and purity checked with HPLC and masspectrophotometry. Methods of peptide synthesis, HPLC purification and mass spectrophotometry are commonly known to those skilled in these art. Inhibitor peptides and inhibitor recep-

tors peptides are also produced in recombinant E. coli or yeast expression systems, and purified with column chromatography.

Different peptide fragments of the intact inhibitor molecule can be synthesized for use in several applications including, but not limited to the following; as antigens for the development of specific antisera, as agonists and antagonists active at inhibitor binding sites, as peptides to be linked to, or used in combination with, cytotoxic agents for targeted killing of cells that bind the inhibitor. The amino acid sequences that comprise these peptides are selected on the basis of their position on the exterior regions of the molecule and are accessible for binding to antisera. The amino and carboxyl termini of the inhibitor, as well as the mid-region of the molecule are represented separately among the fragments to be synthesized.

These peptide sequences are compared to known sequences using protein sequence databases such as GenBank, Brookhaven Protein, SWISS-PROT, and PIR to determine potential sequence homologies. This information facilitates elimination of sequences that exhibit a high degree of sequence homology to other molecules, thereby enhancing the potential for high specificity in the development of antisera, agonists and antagonists to the inhibitor.

Inhibitor and inhibitor derived peptides can be coupled to other molecules using standard methods, eg. labelling or cell type targeting or cytotoxic molecules. The amino and carboxyl termini of the inhibitor both contain tyrosine and lysine residues and are isotopically and nonisotopically labeled with many techniques, for example radiolabeling using conventional techniques (tyrosine residues- chloramine T, iodogen, lactoperoxidase; lysine residues- Bolton-Hunter reagent). These coupling techniques are well known to those skilled in the art. Alternatively, tyrosine or lysine is added to fragments that do not have these residues to facilitate labeling of reactive amino and hydroxyl groups on the peptide. The coupling technique is chosen on the basis of the functional groups available on the amino acids including, but not limited to amino, sulfhydral, carboxyl, amide, phenol and imidazole. Various reagents used to effect the-

se couplings include among others, glutaraladehyde, diazotized benzidine, carbodiimide, and p-benzoquinone. Inhibitor peptides are chemically coupled to isotopes, enzymes, carrier proteins, cytotoxic agents, fluorescent molecules, chemiluminescent, bioluminescent and other compounds for a variety of applications. The efficiency of the coupling reaction is determined using different techniques appropriate for the specific reaction. For example, radiolabeling of an inhibitor peptide with ^{125}I is accomplished using chloramine T and $Na^{125}I$ of high specific activity. The reaction is terminated with sodium metabisulfite and the mixture is desalted on disposable columns. The labeled peptide is eluted from the column and fractions are collected. Aliquots are removed from each fraction and radioactivity measured in a gamma counter. In this manner, the unreacted $Na^{125}I$ is separated from the labeled inhibitor peptide. The peptide fractions with the highest specific radioactivity are stored for subsequent use such as analysis of the ability to bind to inhibitor antisera.

Another application of peptide conjugation is for production of polyclonal antisera. For example, inhibitor peptides containing lysine residues are linked to purified bovine serum albumin using glutaraldehyde. The efficiency of the reaction is determined by measuring the incorporation of radiolabeled peptide. Unreacted glutaraldehyde and peptide are separated by dialysis. The conjugate is stored for subsequent use.

Antiserum specific for the inhibitor, inhibitor analogs, peptide fragments of the inhibitor and the inhibitor receptor can be generated. After peptide synthesis and purification, both monoclonal and polyclonal antisera are raised using established techniques known to those skilled in the art. For example, polyclonal antisera may be raised in rabbits, sheep, goats or other animals. Inhibitor peptides conjugated to a carrier molecule such as bovine serum albumin, or inhibitor itself, is combined with an adjuvant mixture, emulsified and injected subcutaneously at multiple sites on the back, neck, flanks, and sometimes in the rootpads. Booster injections are made at regular intervals, such as every 2 to 4 weeks. Blood samples are obtained by venipuncture, for example using the marginal ear veins after dilation, approximately 7 to 10 days after each injection. The blood samples are allowed to clot overnight at 4^0C and are centrifuged at ap-

proximately 2400 X g at 4⁰C for about 30 minutes. The serum is removed, aliquoted, and stored at 4⁰C for immediate use for subsequent analysis.

All serum samples from generation of polyclonal antisera or media samples from production of monoclonal antisera are analyzed for determination of antibody titer. Titer is established through several means, for example, using dot blots and density analysis, and also with precipitation of radiolabeled peptide-antibody complexes using protein A, secondary antisera, cold ethanol or charcoal-dextran followed by activity measurement with a gamma counter. The highest titer antisera are also purified on affinity columns which are commercially available. Inhibitor peptides are coupled to the gel in the affinity column. Antiserum samples are passed through the column and anti-inhibitor antibodies remain bound to the column. These antibodies are subsequently eluted, collected and evaluated for determination of titer and specificity.

The highest titer inhibitor-specific antisera is tested to establish the following; a) optimal antiserum dilution for highest specific binding of the antigen and lowest non-specific binding, b) the ability to bind increasing amounts of inhibitor peptide in a standard displacement curve, c) potential cross-reactivity with related peptides and proteins of related species, d) ability to detect inhibitor peptides in extracts of plasma, urine, tissues and in cell culture media.

Kits for measurement of inhibitor, and the inhibitor receptor, are also contemplated as part of the present invention. Antisera that possess the highest titer and specificity and can detect inhibitor peptides in extracts of plasma, urine, tissues, and in cell culture media are further examined to establish easy to use kits for rapid, reliable, sensitive, and specific measurement and localization of inhibitor. These assay kits include but are not limited to the following techniques; competitive and non-competitive assays, radioimmunoassay, bioluminescence and hemiluminescence assay, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including ELISA, microtiter plates, antibody coated strips or dipsticks for rapid monitoring of urine or blood, and immunocytochemistry. For each kit the range, sensitivity, precision, reliability, specificity and reproducibility of the assay are established. Intraassay

and interassay variation is established at 20%, 50% and 80% points on the standard curves of displacement or activity.

One example of an assay kit commonly used in research and in the clinic is a radioim-munoassay (RIA) kit. An inhibitor RIA is illustrated below. After successful radioiodi-nation and purification of inhibitor or an inhibitor peptide, the antiserum possessing the highest titer is added at several dilutions to tubes containing a relatively constant amount of radioactivity, such as 10,000 cpm, in a suitable buffer system. Other tubes contain buffer or preimmune serum to determine the non-specific binding. After incu-bation at 4°C for 24 hours, protein A is added and the tubes are vortexed, incubated at room temperature for 90 minutes and centrifuged at approximately 2000 - 2500 X at 4°C to precipitate the complexes of antibody bound to labeled antigen. The supernatant is removed by aspiration and the radioactivity in the pellets counted in gamma counter. The antiserum dilution that binds approximately 10% to 40% of the labeled peptide after subtraction of the non-specific binding is further characterized.

Next, a dilution range (approximately 0,1 pg to 10 ng) of the inhibitor peptide used for development or the antiserum is evaluated by adding known amounts of the peptide to tubes containing radiolabeled peptide and antiserum. After an additional incubation period, for example, 24 to 48 hours, protein A is added and the tubes centrifuged, su-pernatant removed and the radioactivity in the pellet counted. The displacement of the binding of radiolabeled inhibitor peptide by the unlabeled inhibitor peptide (standard) provides a standard curve. Several concentrations of other inhibitor peptide fragments, inhibitor from different species, and homologous peptides are added to the assay tubes to characterize the specificity of the inhibitor antiserum.

Extracts of various tissues, including but not limited to primary and secondary tumors, Lewis lung carcinoma, cultures of inhibitor producing cells, placenta, uterus, and other tissues such as brain, liver, and intestine, are prepared. After lyophilization or Speed Vac of the tissue extracts, assay buffer is added and different aliquots are placed into the RIA tubes. Extracts of inhibitor producing cells produce displacement curves that

are parallel to the standard curve, whereas extracts of tissues that do not produce inhibitor do not displace radiolabeled inhibitor from the inhibitor. In addition, extracts of urine, plasma, and cerebrospinal fluid from animals with Lewis lung carcinoma are added to the assay tubes in increasing amounts. Parallel displacement curves indicate the utility of the inhibitor assay to measure inhibitor in tissues and body fluids.

Tissue extracts that contain inhibitor are additionally characterized by subjecting aliquots to reverse phase HPLC. Eluate fractions are collected, dried in Speed Vac, reconstituted in RIA buffer and analyzed in the inhibitor RIA. The maximal amount of inhibitor immunoreactivity is located in the fractions corresponding to the elution position of inhibitor.

The assay kit provides instructions, antiserum, inhibitor or inhibitor peptide and possible radiolabeled inhibitor and/or reagents for precipitation of bound inhibitor-inhibitor antibody complexes. The kit is useful for the measurement of inhibitor in biological fluids and tissue extracts of animals and humans with and without tumors.

Another kit is used for localization of inhibitor in tissues and cells. This inhibitor immunohistochemistry kit provides instructions, inhibitor antiserum, and possibly blocking serum and secondary antiserum linked to a fluorescent molecule such as fluorescein isothiocyanate, or to some other reagent used to visualize the primary antiserum. Immunohistochemistry techniques are well known to those skilled in the art. This inhibitor immunohistochemistry kit permits localization of inhibitor in tissue sections and cultured cells using both light and electron microscopy. It is used for both research and clinical purposes. For example, tumors are biopsied or collected and tissue sections cut with a microtome to examine sites of inhibitor production. Such information is useful for diagnostic and possibly therapeutic purposes in the detection and treatment of cancer. Another method to visualize sites of inhibitor biosynthesis involves radiolabeling nucleic acids for use in in situ hybridization to probe for inhibitor messenger RNA. Similarly, the inhibitor receptor can be localized, visualized and quantitated with immunohistochemistry techniques.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art.

EXAMPLE 1

Methods

1. Preparation of kringle 1-5 (K1-5) from human plasminogen

Human plasminogen was prepared from pooled human plasma by lysine-Sepharose chromatography as previously described (Cao et al., 1996, JBC, 271, 29461-29467). Purified human plasminogen was incubated in an alkaline solution. The digested mixture was loaded in a lysine-Sepharose column and K1-5 was eluted with a 0-25 (-aminocaproic acid gradient. The K1-5 fraction was further purified on a Sephacryl-S200 column (2,5 x 70 to remove trace amounts of plasminogen contamination. The molecular weight was 55, 000 Dalton verified on SDS gel electrophoresis. Microsequencing analysis revealed that K1-5 consists of a fragment corresponding to plasminogen Lys 77-Arg 529.

2. Endothelial cell proliferation assay

Bovine capillary endothelial cells were maintained in DMEM with 10% heat-inactivated BCS, antibiotics and 3 ng/ml recombinant human bFGF. Cells were washed with PBS and dispersed in a 0,05% solution of trypsin. A cell suspension was made with DMEM, 10% BCS, 1% antibiotics and the concentration was adjusted to 25,000 cells/ml after hemocytometer count. Cells were plated onto gelatinized 24-well culture plates (0,5 ml/well) and were incubated (370C in 10% CO2) for 14h. The medium was replaced with 0,25 ml DMEM, 5% BCS and the test samples at different concentrations were applied. After 20 min incubation, medium and bFGF were added to each well to obtain a final volume of 0,5 ml of DMEM, 5% BCS, 1% antibiotics and

1 ng/ml bFGF. After 72h incubation, cells were dispersed in trypsin, resuspended in Hematall and counted by Coulter counter.

EXAMPLE 2

1. Preparation of K1-5 from human plasminogen.

Human plasminogen was prepared from pooled human plasma by lysine-Sepharose chromatography as previously described (Cao et al., 1996, JBC, 271, 29461, 29467). approximately 40 mg of plasminogen in 4 ml of glycine buffer (pH 10.5) was incubated with immobilized urokinase-activated plasmin (10 Mol of plasminogen/1 Mol plasmin) for 24 h at 25°C. The digested mixture was loaded in a lysine-Sepharose column (1.0 x 30 cm), and K1-5 was eluted with a 0-25 ε-aminocaproic acid gradient.The K1-5 fraction was further purified on a Sephadex G-75 column (2.6 x 90). The molecular weight was 55,000 verified on SDS gel electrophoresis. Microsequencing analysis revealed that K1-5 consists of a fragment corresponding to plasminogen Lys 77 (78) - Arg 529 (530).

2. Endothelial cell proliferation assay.

Bovine capillary endothelial cells were maintained in DMEM with 10% heat-inactivated BCS, antibiotics and 3 ng/ml recombinant human bFGF. Cells were washed with PBS and were dispersed in a 0.05% solution of trypsin. A cell suspension was made with DMEM, 10% BCS, 1% antibiotics and the concentration was adjusted to 25,000 cells/ml after hemocytometer count. Cells were plated onto gelatinized 24-well culture plates (0.5 ml/well) and were incubated (370C in 10% CO2) for 14 h. The medium was replaced with 0.25 ml DMEM, 5% BCS and the test samples at different concentrations were applied. After 20 min incubation, medium and bFGF were added to each well to obtain a final volume of 0.5 ml of DMEM, 5% BCS, 1% antibiotics and 1 ng/ml bFGF. After 72 h incubation, cells were dispersed in trypsin, resuspended in Hematall and counted by Coulter counter.

3. Chick embryo chorioallantoic membrane assay (CAM).

3-d-old fertilized white Leghorn eggs were cracked, and chick embryos with intact yolks were placed in 100 x 20 mm plastic petri dishes. After 3-d of incubation in 3% CO_2 at 37(C, a disk of methylcellulose containing 10 µg of K1-5 was implanted on the CAM of each individual embryo. After 48 h incubation, embryos and CAMs were analyzed for the formation of avascular zones by a stereoscope.

4. Tumor studies in mice.

Male 6-week-old C57B16/J mice were used for tumor studies. Murine T241 fibrosarcoma cells (1 x 10⁶) growing in a log phase were harvested, resuspended in PBS, and implanted subcutaneously in the midline dorsum of each animal in a volume of 100 µl. Three mice were used in each treatment and control group. Subcutaneous injections with either 100 µl of PBS or 100 µl of K1-5 were begun shortly after injection of tumor cells and continued once a day, for a total of 20 days. Visible tumors were present after 24 h. Primary tumors were present after 24 h. Primary tumors were measured blindly using digital calipers on the days indicated.

EXAMPLE 3

Materials and methods

Reagents, cells, and animals. Bovine capillary endothelial (BCE) cells were kindly provided by Dr. Judah Folkman and maintained in DME medium with 10% heat-inactivated bovine calf serum, antibiotics, and 3 ng/ml of recombinant human fibroblast growth factor-2(FGF-2, bFGF) (Scios Nova Inc. Mountainview, CA). Recombinant human fibroblast growth factor-1 (FGF-1) was expressed and purified as previously described (28. Cao, Y. & Pettersson, R. F. (1990) Growth Factors 3, 1-13.) Murine T241 fibrosarcoma tumor cells were maintained in culture in DME medium supplemented with 10% FCS (Hyclone Laboratories, Logan UT) and antibiotics. Male 5-6-wk-old C57B16/J mice (MTC, Karolinska Institute, Sweden) were acclimated and caged in groups of six or less. Animals were anaesthetized in a methoxyflurane chamber before all rpocedures and killed with a lethal dose of methoxyflurane. All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Board.

<u>Preparation of proteolytic fragments of human K1-5 and angiostatin.</u>

Intact, N-terminal Glu^1-Pgn was purified from outdated and citrated human blood plasma by affinity chromatography on L-Lysine-Sepharose, with a gradient of the ligand, 6-aminohexanoic acid (2. Cao, Y., Ko, R.-W., Davidson, D., Schaller, J., Marti, D., Sohndel, S., McCance, S. G., O'Reilly, M.S., Llinas, M. & Folkman, *J. (1996) J. Chem.* 271, 29461-19467.) Approximately 40 mg of purified Glu^1-Pgn in 4 ml of 0.1 M glycine buffer (pH 10.5) was incubated at 25°C with immobilized urokinase-activated plasmin (10 M Pgn in 1 M plasmin) for 14 h (29. Wu, H. L., Chang, B. I., Wu, D. H., Chang, L. C., Gong, C. C., Lou, K. L. & Shi, G. Y. (1990) *J. Biol Chem.* 265, 19658-19664.) After incubation, the sample was applied to a lysine-Sepharose column (1.0 x 30 cm) pre-equilibrated and washed with 1.0 M phosphate buffer (pH 8.0). A peak containing micro-Pgn was detected in the flow-through fraction. K1-5 was eluted from the column with a 0-25 m M linear gradient of 6-aminocapronic acid (Sigma). The protein fraction containing K1-5 was further purified with a Sephadex G-75 column (2.6 x 90 cm). The purified K1-5 was dialyzed against distilled H$_2$O and lyophilozed. The purity of K1-5 was analyzed by SDS-PAGE. The purified K1-5 was analyzed by N-terminal and C-terminal sequencing. Human angiostatin was prepared by diestion of Pgn with procine elastase and purified by affinity chromatography on lysine-Sepharose 4B as previously described (2. Cao, Y., Ko, R.-W., Davidson, D., Schaller, J., Marti, D., Sohndel, S., McCance, S. G., O'Reilly, M.S., Llinas, M. & Folkman, *J. (1996) J. Biol. Chem.* 271, 29461-19467.)

<u>Endothelial Cell Proliferation Assay.</u> Bovine capillary endothelial (BCE) cells were isolated as described previously (30. Folkman, J., Haudenschild, C. C. & Zetter, B. R. (1979) *Proc. Natl. Acad. Sci. USA.* 76, 5217-5121.) BCE cells were maintained in DME medium containing 10% heat-inactivated bovie calf serum (BCS) and 3 ng/ml of recombinant human bFGF. Cells growing in gelatinized 6-well plates were dispersed in 0.05% solution and resuspended with DME medium containing 10% BCS. Approximately 10,000 cells in 0.5 ml were added to each gelatinized wells of 24-well plates and incubated at 37°C for 24 h. The medium was replaced with 0.5 ml fresh DME me-

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dium containing 5% BCS and samples of kringle structures in triplicates were added to each well. After 30 min incubation, bFGF was added to a final concentration of 1 ng/ml. After 72 h incubation, cells were trypsinized, resuspended in Isoton II solution (Coulter Electronics Ltd. Beds, England) and counted with Coulter counter.

Chick embryo chorioallantoic membrane (CAM) assay. The CAM assay was carried out as previously described (31. Cao., Y., Chen, C., Weatherbee, J. A., Tsang, M. & Folkman, J. (1995) *J. Exp. Med.* 182, 2069-2077.) (32. Nguyen, M., Shing, Y. & Filkman, . (1994) *Microvascular Res.* 47, 31-40.) Three-day-old fertilized white Leghorn eggs (OVA Production, Sörgården, Sweden) were cracked and chick embryos with intact yolks were carefully placed in 20 x 100 mm plastic petri-dishes. After 48 h incubation in 4% CO_2 at 37°C, disks of methylcellulose containing various concentrations of K1-5 dried on nylon meshes (4 x 4 mm/each) were implanted on the CAMs of individual embryos. The nylon mesh disks were made by desiccation of 20 µl of 0.45% methylcellulose (in H_2O). After 48-72 hours of incubation, embryos and CAMs were examined under a stereoscope for the formation of avascular zones in the field of the implanted disks.

Mouse corneal micropocket assay. The mouse corneal assay was performed as previously described (27. Cao, Y., O'Reilly, M. S., Marshall, B., Flynn, E., Ji, W. R. & Folkman, J. (1998) J. Clin. Invest. 101, 1055-1063. 31. Cao, Y., Chen, C., Weatherbee, J. A., Tsang, M. & Folkman, J. (1995) *J. Exp. Med.* 182, 2069-2077. 34. Jain, R. K., Schlenger, K., Hockel, M. & F., Yuan. (1997) *Nature Med.* 3, 1203-1208). 7-wk-old male C57B16/J mice, each ≈20 g, were divided into three groups. Control group (n=5) received daily subcutaneous injections of 100 µl of phosphate-buffered saline, including the day before pellet implantation. The other two groups (n=5) were subcutaneously injected once daily with K1-5 and angiostatin in 100 µl of PBS at the dose of 2 mg/kg, including pretreatment with the same dose the day before corenal implantation. Corneal micropockets were created with a modified von Craefe cataract knife in both eyes of each mice. Into each pocket, a pellet containing approximately 80 ng of FGF and sucrose aluminium sulfate (Bukh Meditec., Copenhagen, Denmark) coated with

hydron polymer type NCC (IFN Sciences, New Brunswick, NJ) was implanted. The pellet was positioned approximately 1.5 mm from the corneal limbus. The maximal vessel length, clock hours and the vascular area of corneas of all mice were measured, by means of a slit-lamp stereo-microscope at a magnification of 15, on the sixth day after corneal implantation.

Tumor studies in mice. Male 6-wk-old C57B16/J mice were used for tumor studies. Approximately 1 x 10^6 murine T241 frbrosarcoma cells growing in log phase were harvested, resuspended in PBS, and a single cell solution in a volume of 100 µl was implanted subcutaneously in the middle dorsum of each animal. 5 mice were used in the treated and control groups, respectively. Systemic treatment by subcutaneous injections with either 100 µl of PBS or 2-2.5 mg/kg of K1-5 and angiostatin in PBS was begun shrotly after implantation of tumor cells and continued once daily for a total of 18-20 treatments. Visible tumors were present 72 h after implantation. Primary tumors were measured using digital calipers on the days indicated. Tumor volumes were calculated according to the formula: $width^2$ x length x 0.52 as previously reported (27. Cao, Y., O'Reilly, M. S., Marshall, B., Flynn, E., Ji, W. R. & Folkman, J. (1998) J. Clin. Invest. 101, 1055-1063).

Histology. Tumor bearing C57B16/J animals were killed by an overdose methoxyflurane on day 20 after tumor cell implantation, and viable tumor tissues were resected and fixed with 4% formalin in phosphate-buffed saline for 24 h. Tissues were imbedde in paraffin according to standard histological procedures (27. Cao, Y., O'Reilly, M. S., Marshall, B., Flynn, E., Ji, W. R. & Folkman, J. (1998) J. Clin. Invest. 101, 1055-1063). The sections of 5 µm thickness were processed and stained with a rabbit anti-human von Willebrand factor (Dako Corp., Carpinteria, CA) antibody as previously described (27. Cao, Y., O'Reilly, M. S., Marshall, B., Flynn, E., Ji, W. R. & Folkman, J. (1998) J. Clin. Invest. 101, 1055-1063.). Microvessels were counted per high power field (x 40) in 5 random fields of tumors from 4 mice of each group.

Results

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Identification and characterization of proteolytic K1-5. Serine proteases have recently been reported to be involed in the generation of angiogenesis inhibitors such as angiostatin (35. Gately, S., Twardowski, P., Stack, m. S., Patrick, M., Boggio, L., Cundiff, D. L., Schnaper, H. W., Madison, L., Volpert, O., Bouck, N., Enghild, J., Kwaan, H. C. & Soff, G. A. (1997) *Canser Res.* 56, 4887-4890). Plasmin is a serine protease and angiostatin is an internal fragment of Pgn. To further study the role of the Pgn-plasmin system in the control of the switch of angiogenesis, human Glu^1-Pgn was digested with urokinase-activated plasmin as described under "Materials and Methods" (Fig. 1A). The proteolytic fragments were purified by affinity chromatography on lysine-Sepharose, followed by gel filtration through a Sephadex G-75 column. SDS-gel eletrophoresis showed that a proteolytic fragment with a molecular mass of 55 kDa was purified to homogeneity (1B). Amino- and carboxyl-terminal sequencing analyses showed that the cleavage sites of plasmin were dibasic amino acids between Lys^{76} and Lys^{77} at the NH_2 terminus, and between Arg^{529} and Lys^{530} at the COOH terminus, respectively (1A). Thus, this fragment contains the intact K1-4 and most K5 domains (K1-5) of Pgn, termed K1-5.

Inhibition of endothelial cell proliferation by K1-5. To investigate if K1-5 could inhibit endothelial cell proliferation, purified proteolytic K1-5 was incubated with BCE cells stimulated by FGF-2 at the concentration of 1 ng/ml. K1-5 inhibited BCE cell growth in a dose-dependent manner (Fig. 2A). The concentration of K1-5 required to reach 50% inhibiton (IC_{50}) was approximately 50 pM and the maximal inhibiton was observed at the concentration of 200 pM (2B). Higher concentrations did not further increase the inhibitory effect of K1-5 (2B and C) on the cells. The IC_{50} appears to be at least 50-fold lower than that of proteolytic human angiostatin. In the presence of K1-5, the morphology of BCE cells appeared to be similar to that of untreated cells. In addition, cell proliferation ca be rescued with FGF-2 stimulation after removal of K1-5 at a high concentration of 132 nM (2D). These data suggested that K1-5 was not toxic to BCE cells even at a high concentration. The anti-proliferative effect of K1-5 appeared to be endothelial cell specific since the concentration required for maximal inhibition of BCE cell proliferation was not inhibitory on several non-endothelial cells lines, inclu-

ding primary human firboblasts, human retina pigment cells, NIH3T3 fibroblasts, rat smooth muscle cells, BHK cells and T241 firbrosarcoma cells (data not shown).

Synergistic inhibition of BCE cell proliferation by angiostatin and K5. K5 of human and mouse Pgn has recently been reported to be a potent endothelial inhibitor (23. Cao, Y., Chen, A., An, S. S. A., Ji, R. W. D., Davidson, D., Cao, Y. M. & Llinas, M. (1997) J. Biol. Chem. 272, 22924-22928). Since the structure of K1-5 contains angiostatin (K1-4) and K5, the enhancing effect of angiostatin and K5 on inhibition of BCE cell proliferation was tested. At the concentration of 1 nM, proteolytic human angiostatin did not significantly inhibit BCE cell proliferation, and approximately less than 50% inhibitory effect of BCE cell proliferation by human proteolytic K5 was observed (Fig. 3A and B). However, a synergistic efficacy of inhibition, which was approximately the maximal inhibition (>95%), was detected when both fragments at the concentration of 1 nM were added together to BCE cells (3A and B). Interestingly, this synergistic inhibitory efficacy is comparable to that produced by K1-5 alone (3B). High concentrations of K5 and angiostatin did not significantly show synergistic effects on suppression of BCE cell proliferation because K5 alone displayed nearly a maximal inhibition with increased concentrations (data not shown). These results demonstrate that angiostatin and K5 synergisticalloy suppress BCE cell proliferation.

Inhibition of angiogenesis in the CAM. To study the in vivo antiangiogenic activity, proteolytic human K1-5 was tested on the CAM. At concentrations of 5-25 µg/disk, K1-5 inhibited new embryonic blood vessel growth as measured by the formation of avascular zones (Fig. 4B, the area marked with curved arrows). Within the avascular zone, a large number of newly formed blood vessels were regressed. The measured inhibition was dose dependent (4C) over the ragne of 5-25 µg/embryo, and no obvious inflammation was detected. No avascular zones were found in the control embryos implanted with disks containing PBS alone (4A and C). These results demonstrate that K1-5 is able to suppress angiogenesis in embryos.

Suppression of mouse corneal neovascularization. To further investigate the antiangiogenic activity of K1-5 *in vivo* and to directly compare the angiangigenic efficacy of K1-5 with that of angiostatin, the inhibitory effect of systemic administration of K1-5 and angiostatin on FGF-induced corneal neovascularization was studied. The rigorous antiangiogenic assay requires a putative angiogenesis inhibitor to be administrated systemically (e.g. subcutaneous injections or intraperitoneal) in order to examine its capacity to suppress 80 ng of FGF-induced corneal neovascularization. Systemic treatment of mice with K1-5 by subcutaneous injections at the concentration of 2 mg/kg/once daily significantly blocked the FGF-induced corneal neovascularization (Fig. 5C). The length (5D) and clock hours (5E) of corneal circumferential neovascularization were inhibited by 50-70% in 10 corneas of 5 individual mice. The density of corneal vessels in the K1-5-treated animals (5C) was also markedly reduced as compared with that of control animals treated with PBS (5A). About 80% reduction of the area of corneal neovascularization was observed in the K1-5-treated group as compared with that of the control group treated with PBS alone (5F). In contrast, angiostatin at the same dose did not significantly inhibit the FGF-induced corneal vascularization (5B, D, E and F) as compared with the PBS-treated group (A, D, E, and F). Thus, K1-5-displayed a more potent antiangiogenic effect than K1-4 in the rigorous *in vivo* model. The treated mice did not experience weight loss or unusual behavior over the course of the treatment, indicating that K1-5 was not toxic at the dose used in our experiments.

Suppression of primary tumor growth by systemic administration of K1-5. Since K1-5 inhibited neovascularization *in vivo* and tumor growth requires angiogenesis (9. Folkman, J. (1995a) *Nature Med.* 1, 27-31), we determined the antitumor activity of K1-5. Proteolytic human K1-5 was used to systemically treat C57B16/J mice bearing subcutaneously implanted primary T241 fibrosarcomas. K1-5 at the dose of 2.5 mg/kg/day was subcutaneously injected once daily in the ventral abdomen, whereas subcutaneous tumors were growing in the midline dorsum of each mouse. This daily systemic administration of K1-5 resulted in a significant suppression of the growth of primary tumors during the 20-d treatment course (Fig. 6A and B). At day 20 after treatment, an average

of over 65% suppression of primary tumor growth was observed in the K1-5-treated mice (n=5). In contrast, tumors grew rapidly to sizes >7500 mm^3 in the saline-treated animals (n=5) during the same 20-d treatment period (6B), leading to the demise of all mice within 5 wk after tumor implantation. The K1-5-treated mice showed no weight loss and unusual behaviour over the course of treatment.

In order to directly compare the antitumor efficacy of K1-5 with that of angiostatin, we performed the side-by-side comparison experiment using the same tumor model system. As shown in Fig. 7, systemic treatment of K1-5 at the concentration of 2 mg/kg/once daily again significantly suppressed the growth of T241 fibrosarcoma in syngeneic C57Bl6/J mice (n=5) by approximately 63% as compared with the control group treated with PBS (n=5). In contrast, angiostatin at the same dose did not significantly block tumor growth (n=5) when compared the PBS-treated group during the 18-d treatment course. We should emphasize that although K1-5 significantly inhibited tumor growth, it did not completely arrest tumors at a dormant stage. The K1-5-treated tumors would eventually grew to large sizes which were comparable to the control tumors treated with PBS or angiostatin, but at a delayed rate.

Suppression of tumor neovascularization. To evaluate the suppressive effect of K1-5 on tumor angiogenesis, tumor tissue sections were immunohistochemically stained with an endothelial specific antibody against von Willebrand factor and microvessels in tumor tissues were randomly counted. Primary tumors were resected at day 20 after systemic treatment with K1-5 treatment. A dramatically decreased microvessel density in tumor tissues was revealed in the K1-5-treated mice (Fig. 8B, C and D) as compared to control tumor tissues treated with PBS (p<0.001) (A and D). Figure 6D represented an average of vessel density of 6 random fields in tumors from 3 mice of each group.

EXAMPLE 4

Preparation of cDNA encoding K1+K5 fusion protein

Both methods described below utilise conventional methodologies well known to the skilled in this field, e.g. such as mentioned above.

(a) Recombinant DNA technology

In order to generate a fusion protein between kringle 5 and kringle kringle 1, the complementary DNA (cDNA) sequence encoding K5 and the cDNA encoding K1 are cloned. The cloning is performed by PCR technology. Once these two part of cDNA fragments have been cloned, they are fused by either PCR or enzymatic ligation. Such fusion has to be in frame in terms of amino acid translation. Once the cDNAs coding for K1 and K5 are fused, the fused cDNA is cloned into expression vectors that can be used to produce recombinant proteins in either mammalian cells, insect cells, yeast cells and bacterial cells.

(b) Chemical fusion

The proteins are fused by use of chemical compounds. Some chemicals allow the fusion of proteins directly. The fusion of kringle structures also includes self fusion of the same kringle structure. For example, a Kringle 5 polymer that displays a more potent effect may be prepared. Thus, these kringle 5 or kringle 1 polymers contain several kringle 5 or kringle 1.

In the present context, it is to be noted the importance of having the information of sequence of the start and end of kringle 1 and kringle 5. In priciple, kringle 1 will include the first animo acid of glutamine (Glu1) of plasminogen and kringle 5 will include the proline 544 (Pro544). This fragment between Glu1-544 will cover everything needed to produce K1-5.

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CLAIMS

1. A composition comprising one or more proteins or peptides which individually or together comprise

(a) an amino acid sequence corresponding to that of one or more of Kringle 1, 2 and 3 of a mammalian plasminogen or anti-angiogenically active peptide fragments or protected derivatives thereof and

(b) an amino acid sequence corresponding to that of a peptide or protein selected from the group consisting of a mammalian plasminogen Kringle 5, its fusion proteins or anti-angiogenically active peptide fragments or protected derivatives thereof, wherein the total molecular weight of the protein and/or peptides (a) + (b) is no more than 50-70kDa as defined by reducing polyacrylamide gel electrophoresis.

2. A composition according to claim 1, wherein the amino acid sequence corresponding to that of one or more of K1, 2 and 3 of a mammalian plasminogen consists of a protein corresponding to the amino acid sequence of a mammalian plasminogen fragment K1, K2, K3, K1-2, K1-3, K2-3, K1-4, K2-4 or K3-4 and is of 45KDa or less as determined by reducing polyacrylamide gel electrophoresis.

3. A composition according to claim 1 or 2, wherein the peptide or protein having amino acid sequence corresponding to that of a mammalian plasminogen Kringle 5, its fusion proteins or an anti-angiogenically active peptide fragment or protected derivative thereof is selected from the group of molecules comprising the amino acid sequence of mammalian plasminogen Kringle 5 Arg530 to Tyr534 and molecules comprising the amino acid sequence of mammalian plasminogen Kringle 5 Pro150 to Tyr 156.

4. A composition according to any one of claims 1-3, wherein the amino acid sequence corresponding to that of a mammalian plasminogen Kringle 5, its fusion proteins or anti-angiogenically active peptide fragments or protected derivatives thereof is selected from the group of molecules comprising the amino acid sequence of mammalian plasminogen Kringle 5 Val454 to Ala543.

5. A composition according to any one of claims 1-4 comprising a single anti-angiogenically active protein, wherein the amino acid sequence corresponding to that of one or more of mammalian Kringles 1, 2 and 3 and that of Kringle 5 are pre-

sent in the same sequence, joined together directly or by a spacer amino acid sequence.

6. A composition according to claim 5, wherein the protein comprises a spacer sequence corresponding to that of a mammalian Kringle 4.

7. A composition according to claim 5 or 6, wherein the protein comprises an amino acid sequence corresponding to that of a mammalian plasminogen Kringles 1 to 5 Lys78 to Arg529.

8. A protein or peptide having an amino acid sequence corresponding to that of a mammalian plasminogen kringle selected from the group consisting of K1, K2, K3, K1-2, K1-3, K1-4, K2-3, K2-4, K3-4, or an anti-angiogenically active peptide fragments of any of these, fused to an amino acid sequence corresponding to that of mammalian plasminogen K5 or an anti-angiogenically active fragment thereof.

9. A protein or peptide according to claim 8 comprising an amino acid sequence corresponding to that of any one of the group consisting of K1 fused to K5, K2 fused to K5, K3 fused to K5, K1-2 fused to K5, K1-3 fused to K5, and K2-3 fused to K5.

10. A nucleic acid, which encodes a protein or peptide according to claim 8 or 9 or a functional fragment or an analogue thereof, or any variant thereof.

11. A nucleic acid which encodes one or more proteins or peptides which individually or together comprise

(a) an amino acid sequence corresponding to that of one or more of Kringles 1, 2 and 3 of a mammalian plasminogen or an anti-angiogenically active fragment thereof and

(b) an amino acid sequence corresponding to that of peptides and proteins selected from the group consisting of a mammalian plasminogen Kringle 5, its fusion proteins or anti-angiogenically active peptide fragments or protected derivatives thereof wherein

the total molecular weight of the encoded protein and/or peptides (a) + (b) is no more than 50-70kDa as defined by reducing polyacrylamide gel electrophoresis.

12. A nucleic acid, which specifically hybridizes, under stringent conditions, to a nucleic acid according to claim 10 or 11.

13. An expression vector wherein it comprises a nucleic acid according to any one of claims 10-12, which preferably is a viral vector.

14. A composition comprising a vector according to claim 13 together with one or more pharmaceutically acceptable carriers and/or excipients.

15. A composition as defined in any one of claims 1-7 or a protein or a peptide as defined in claim 8 or 9 for use as a medicament.

16. Use of a composition as defined in any one of claims 1-7 or a protein or a peptide as defined in claim 8 or 9 in the manufacture of a medicament for treatment of a patient in need of anti-angiogenic therapy.

17. A pharmaceutical or veterinary preparation, which comprises a composition as defined in any one of claims 1-7 or a protein or a peptide as defined in claim 8 or 9 and one or more pharmaceutically acceptable carriers and/or excipients.

18. A pharmaceutical or veterinary preparation, which comprises a composition as defined in any one of claims 1-4 and in which component (a) and component (b) of said composition are present as separate entities combined for administration at the same time, or in sequence as part of the same treatment scheme.

19. A pharmaceutical or veterinary preparation, which comprises a composition as defined in any one of claims 5-7 or a protein or peptide according to claim 8 or 9 in a single dosage form.

20. An antibody raised against a peptide or protein according to claim 8 or 9.

21. A screening assay, wherein a peptide or protein according to claim 8 or 9, a nucleic acid according to any one of claims 10-12 or an antibody according to claim 20 is used to identify a target.

22. A method of diagnosis and/or prognosis, wherein a peptide or protein according to claim 8 or 9, a nucleic acid according to any one of claims 10-12 or an antibody according to claim 20 is used.

23. A kit for performing a method according to Claim 22, which comprises one or more separate compartments for the different components thereof present in a suitable container and preferably written instructions regarding the use thereof.

24. A method of treating a patient in need of anti-angiogenic therapy comprising administering to that patient an effective dose of a composition according to any one of claims 1-7, a protein or peptide according to claim 8 or 9 and/or a pharmaceutical preparation according to any one of claims 17-19.
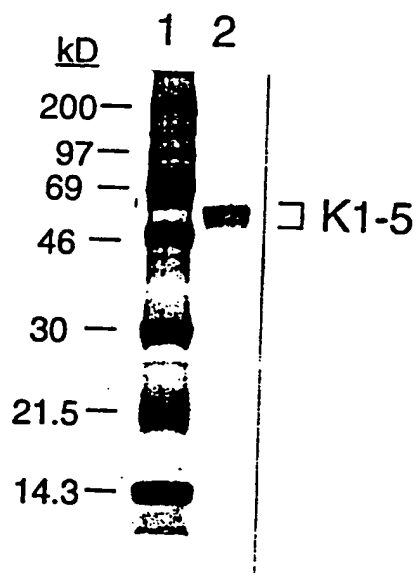
Fig. 1

A
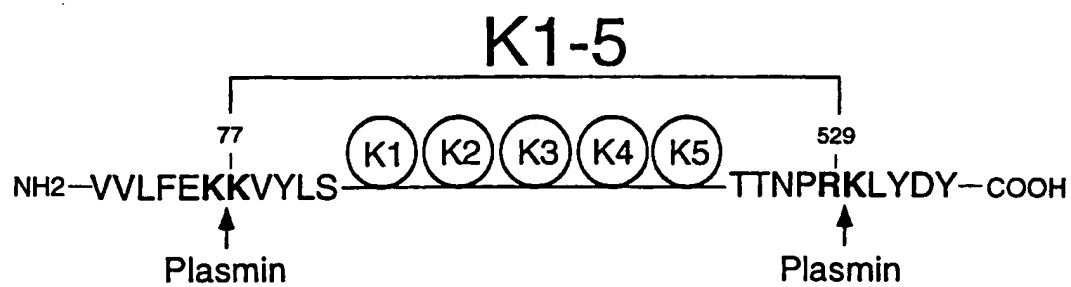
B

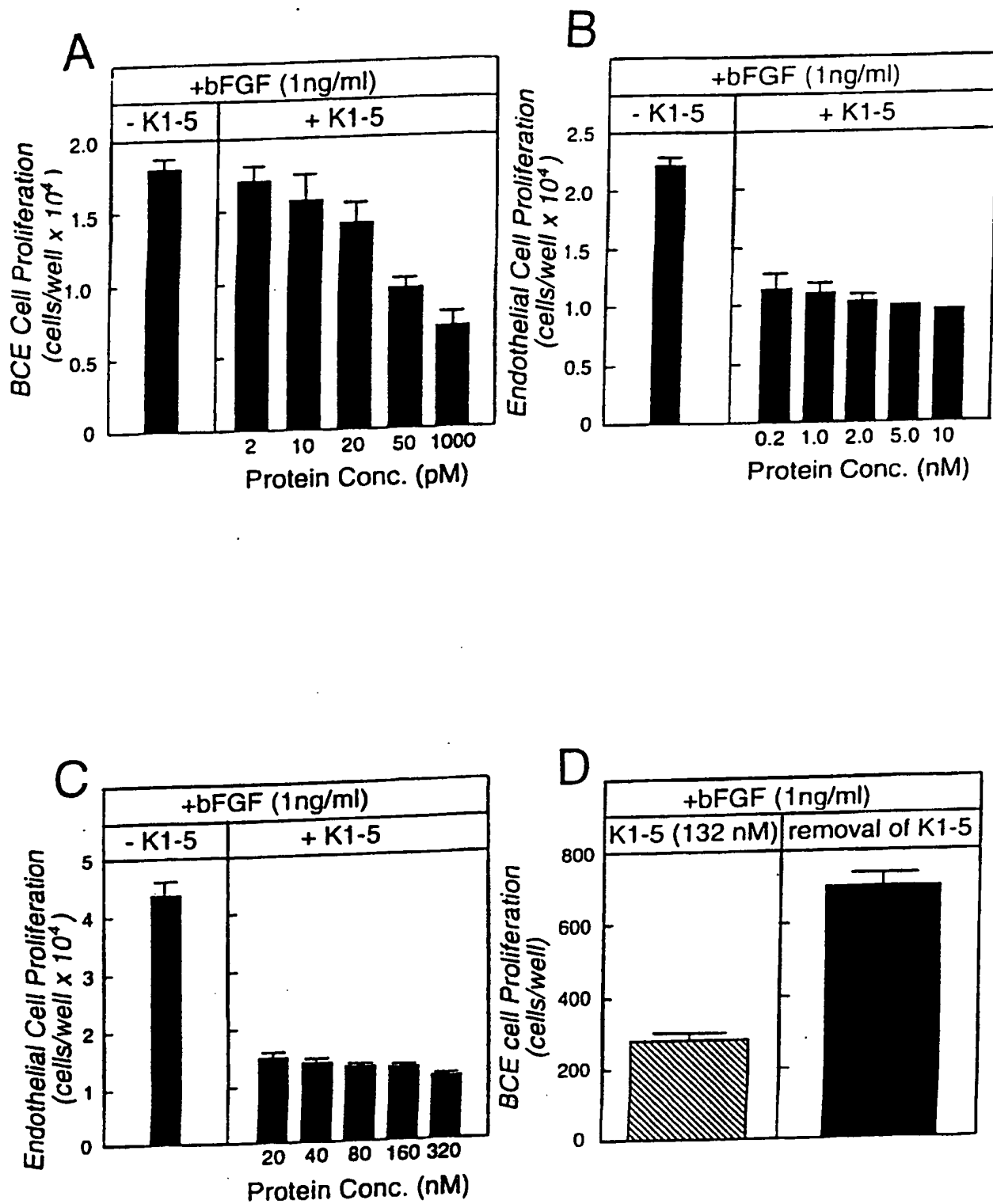Fig. 2

Fig. 3

A bFGF alone

B bFGF + K1-5 (10ng/ml)

C bFGF + K1-5 (100ng/ml)

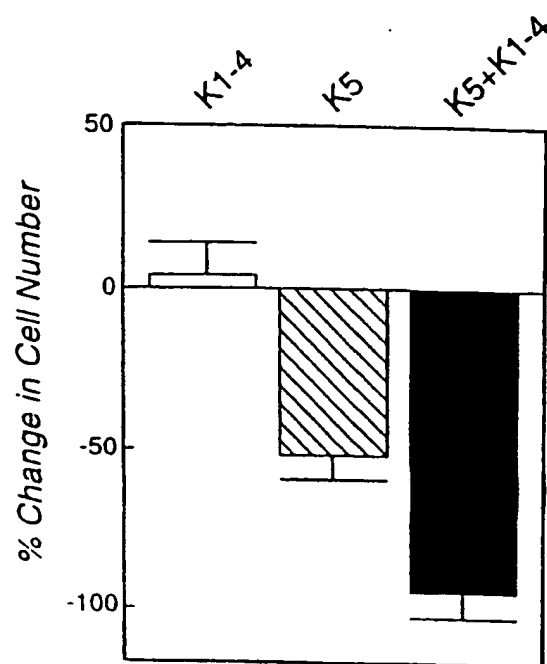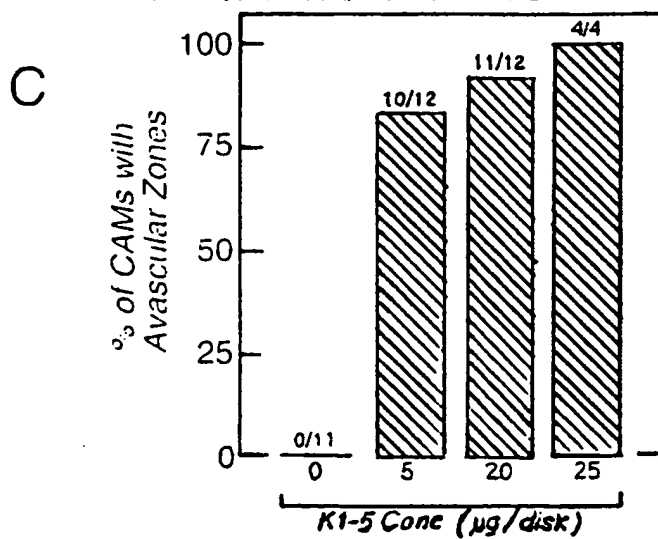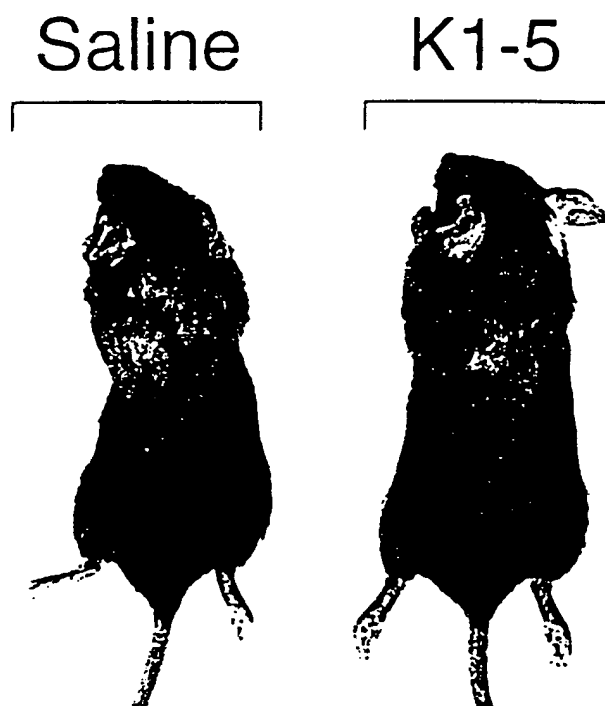D bFGF + K1-5 (500ng/ml)

Fig. 4   4/9

Fig. 5
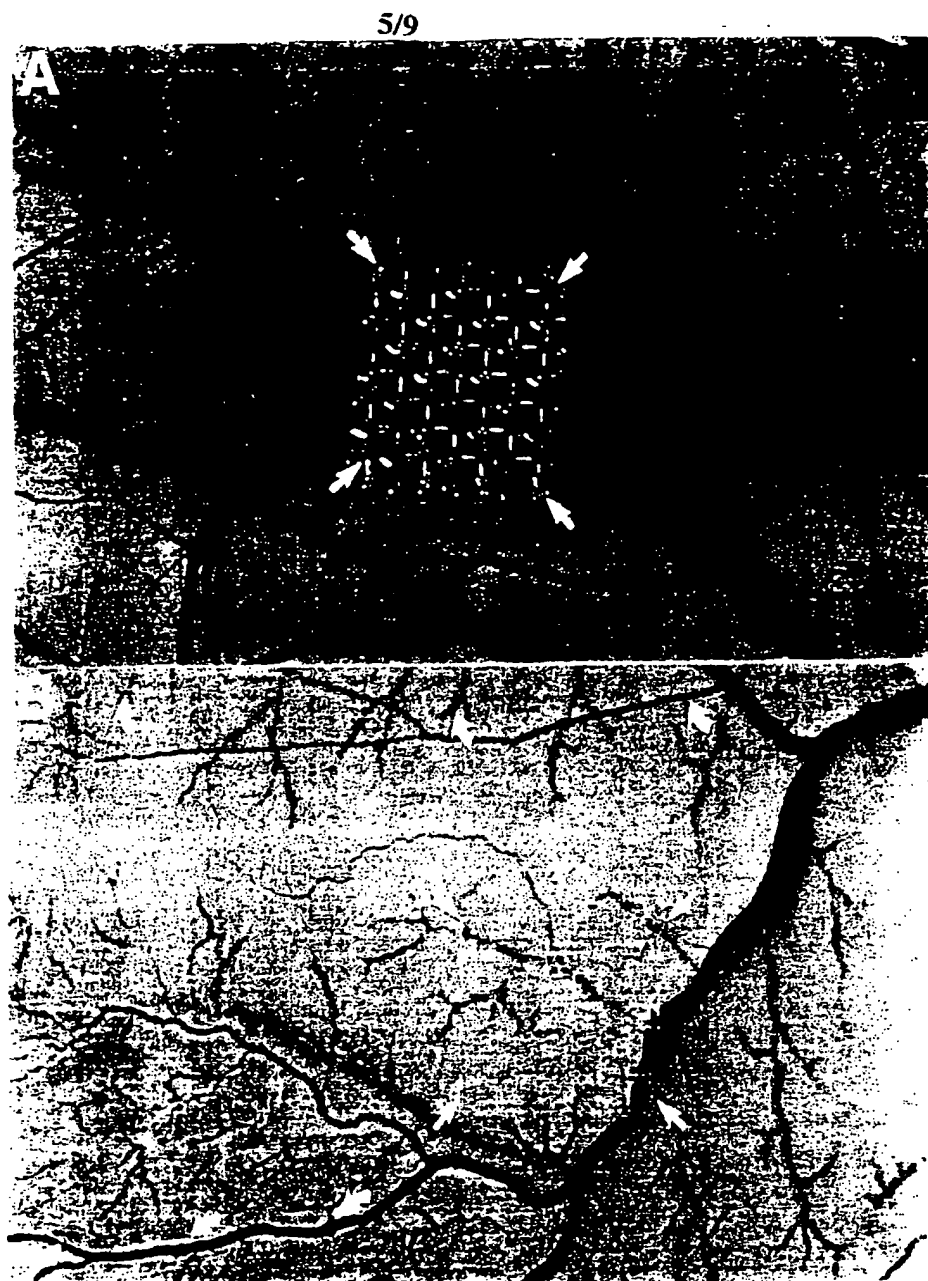
Fig. 6A

Fig. 6B
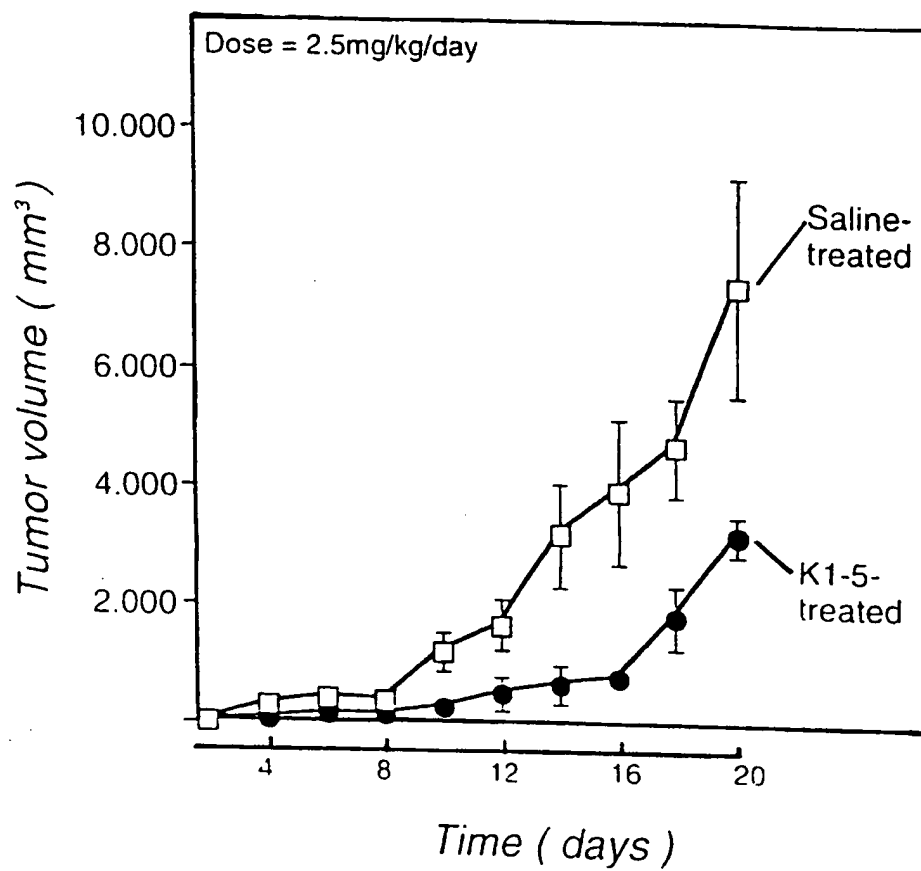
Fig. 7
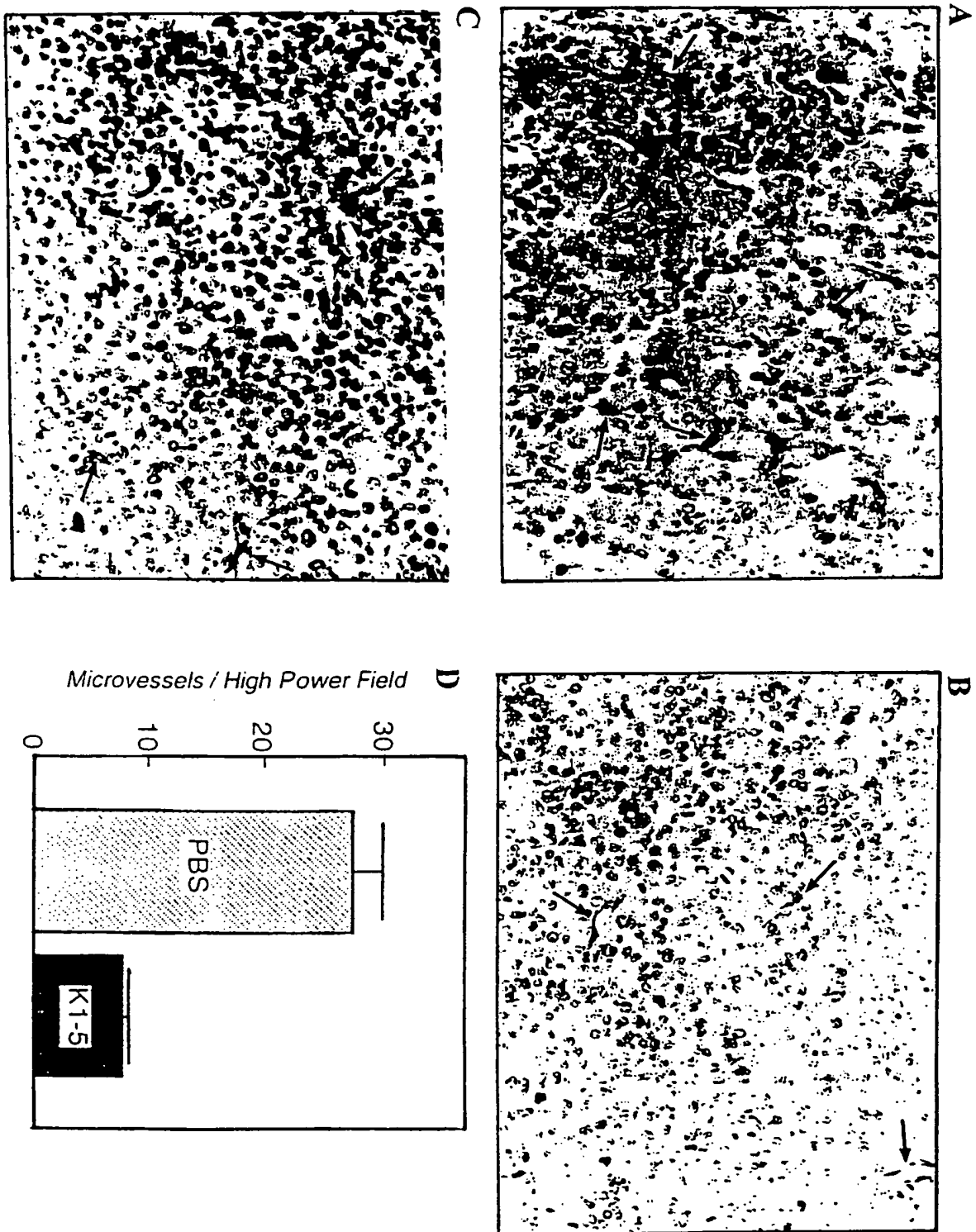
Fig. 8a

Fig. 8b

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 38/43, C12N 9/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K, C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E,X	WO 0031244 A1 (BRISTOL-MYERS SQUIBB COMPANY), 2 June 2000 (02.06.00) --	1-24
X	WO 9854217 A1 (THE CHILDREN'S MEDICAL CENTER CORPORATION), 3 December 1998 (03.12.98), page 17, line 17 - line 19; page 89, line 5 - page 91, line 14; page 93, line 2 - line 5, page 105, line 22 - line 26 and the claims --	1-24
X	WO 9900420 A1 (KAROLINSKA INNOVATIONS AB), 7 January 1999 (07.01.99), page 19, line 22 - page 20, line 2 --	1-24

|X| Further documents are listed in the continuation of Box C. |X| See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 2000	29 -08- 2000
Name and mailing address of the ISA/ **Swedish Patent Offic** Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Carolina Palmcrantz/ELY Telephone No. + 46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 08879512, Medline accession no. 97067211, Cao Y et al: "Kringle domains of human angiostatin. Characterization of the anti-proliferative activity on endothelial cells"; Journal of biological chemistry (UNITED STATES) Nov 15 1996, 271 (46) p 29461-7	1-24
A	Dialog Information Services, File 155, MEDLINE, Dialog accession no.09243240, Medline accession no. 97426443, Cao Y et al: "Kringle 5 of plasminogen is a novel inhibitor of endothelial cell growth"; Journal of biological chemistry (UNITED STATES) Sep 5 1997, 272 (36)	1-24
A	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 07171691, Medline accession no. 93075152, Wu HL et al: "The binding of plasminogen fragments to cultured human umbilical vein endothelial cells"; Biochemical and biophysical research communictions (UNITED STATES) Oct 30 1992, 188 (2) p 703-11	1-24

Box I	**Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **22 , 24**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See extra sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).:

Box II	**Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application. as follows:

1. ☐ As all required additional search fees were timely paid by the applicant. this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee. this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant. this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

CONJUCHEM INC.
R & D TIME SHEET

Employee: Nissab Boudjellab

Week: 11 Dec. - 15 Dec./ 2000

PROJECT	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	TOTAL
CORE						0
CCI-1004						0
OPIOIDS						0
CYTO						0
GLP-1						0
HALL01 (K5)						0
TRIS01 (RSV)						0
TIBC01 (C34)						0
EXPLORATORY	8h	8h				16h
Vacation , Holidays Sick days, Training (please circle)			8h	8h	8h	24h
TOTAL	0	0	0	0	0	**40h**

Employee Signature _____

Supervisor Signature _____

INTERNATIONAL SEARCH REPORT

Claims 22,24 relate to methods of treatment of the human or animal body by therapy/diagnostic methods practised on the human or animal body (Rule 39.1.(iv)). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compound(s)/composition(s).

INTERNATIONAL SEARCH REPORT

| | Information on patent family members | | 02/12/99 | International application No. PCT/SE 00/00719 |

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0031244 A1	02/06/00	NONE	
WO 9854217 A1	03/12/98	AU 7704998 A US 5945403 A	30/12/98 31/08/99
WO 9900420 A1	07/01/99	AU 7951798 A SE 9703057 D	19/01/99 00/00/00